METABOLISM OF 17α-METHYLTESTOSTERONE BY RAT LIVER

by

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INTRODUCTION AND STATEMENT OF PROBLEM

For the last 17 years, it has been known that when testosterone was administered to mammals increased quantities of etiocholanolone and androsterone appeared in the urine (18,19). Experiments with labeled testosterone have indicated that these steroids isolated from the urine are direct metabolites of testosterone. Where the transformation of testosterone takes place in the body of the animal, or the mechanism by which it is converted into these metabolites, has not been completely solved.

These products may be the result of either of two different processes. In order to maintain a constant internal environment, the organism must reduce, remove, or destroy the high physiological activity of excess testosterone; or the physiological action of the hormone may be associated with its conversion to these metabolites. To gain a better understanding of the mechanism by which testosterone is metabolized, a number of experimental approaches have been used. They are (a) implanting pellets of the hormone at different sites in the body of a castrated animal and then assessing androgenic potency by the response of the secondary sex organs, (b) perfusing an organ or organs with a medium containing the androgen, and then isolating the steroids formed, and (c) incubating tissue slices, minces, homogenates, and preparations of cellular

components with the androgen, and then finding the metabolites produced.

An attempt was made by Samuels, et al. (1) to ascertain the role of the liver in testosterone metabolism. They incubated microgram amounts of the steroid, which were completely soluble in the medium used, with liver tissue. Sweat and Samuels (2) concluded that testosterone was probably catabolized by two different routes. One route was activated by DPN and led to the formation of Δ^4-androstene-3,17-dione while the other one was activated by citrate and did not form 17-ketosteroids. It was shown by Levedahl and Samuels (3) that methyltestosterone was metabolized by the route activated by citrate, which destroyed the α-β unsaturation of ring A, but not by the route activated by DPN which oxidized the hydroxyl group at Carbon 17.

The purpose of this investigation was to study further the liver system activated by citrate which destroyed the α-β unsaturation of ring A . Since methyltestosterone was metabolized by this system but not by the one oxidizing the 17 position, it was chosen primarily as the substrate.

HISTORICAL

I. Metabolism of Testosterone:

Androgen biochemistry could be said to have begun with the work of McGee (4). He prepared lipid extracts from bull testis which would consistently cause a resumption of growth of a capon's comb. Earlier work by Berthold (5) had shown that testis transplants would minimize the effects of castration, and Loewy (6) indicated that injections of testicular substance caused comb growth in young capons. However, it was the preparation of an active extract of testis by McGee (4) that started a wave of investigations which culminated in the crystallization of a highly potent androgen from bull testis by David et al. (7). These authors named the crystalline material testosterone. Its structure was established as Δ^4-androstene-17β-ol-3-one when it was synthesized by Ruzicka et al. (8) and Butenandt and Hanisch (9) from cholesterol.

From the time of its isolation, testosterone was considered to be the naturally occurring male hormone even though it had not been isolated from the blood stream. In 1952, West et al. (10) succeeded in isolating it from the spermatic vein blood of dogs. At the present time, the evidence indicates that it is the natural male hormone because (a) it is the most active naturally occurring androgen found (11), (b) it has been isolated from testis (7)(12), (c) it has been isolated from

spermatic vein blood in greater amounts than could be present in peripheral blood (10), and (d) it can be synthesized by testis tissue (13)(14).

Not long after the isolation and characterization of testosterone it was found that an increase in urinary androgens accompanied its administration (15)(16)(17). Callow (18) and Dorfman et al. (19) were able to isolate increased amounts of etiocholanolone and androsterone from the urine of patients receiving large doses of testosterone. Later Dorfman (20) isolated androstane-3β-ol-17-one from the urine of a hypogonadal male given testosterone therapy.

Deuterium labeled testosterone was injected into a normal man by Gallagher et al. (21) and the urinary metabolites were measured. Androsterone and etiocholanolone were found to be the major metabolites, but small quantities of androstane-3, 17-dione and etiocholane-3,17-dione were also present. Only 39 per cent of the injected dose was recovered.

Fukushima et al. (22) compared the metabolites excreted and the percentage of administered dose recovered after a 100 mg. and a 10 mg. injection of deuterium labeled testosterone into normal men. The same labeled steroids reported by Gallagher (21) were isolated. No difference was observed in the ratio of the metabolites excreted; the recovery of the dose administered was 49 per cent of the 10 mg. and 39 per cent of the 100 mg. injection. A difference was noted in the excretion pattern of individuals. One subject produced predominately etiocholanolone

while the others produced predominantly androsterone.

The same group of investigators (23) then infused 16 mg. of testosterone-d-4-C^{14} over a period of 4 hours into a normal man and were able to recover 60 per cent of the dose. West et al. (24) were able to recover 74 per cent of a dose of 250 mg. testosterone infused into a normal man. This is the best recovery yet reported.

Mammals other than man have been given testosterone and the products excreted in the urine determined. Dorfman and Fish (25) injected testosterone propionate into guinea pigs and were able to isolate androstane-3β-ol-one. The same workers (26) also isolated etiocholanolone, androsterone, and Δ^4-androstene-3,17-dione from the urine of chimpanzees receiving testosterone propionate. Monkeys may also produce androsterone when they are given testosterone, as was shown by Horwitt et al. (27).

The experiments just mentioned indicate that the major end products of testosterone metabolism are androsterone and etiocholanolone but give no hint as to where this transformation takes place or the conditions accompanying it. Evidence that the liver is involved first came from Biskind and Mark (28). Pellets of testosterone implanted into the spleen were much less effective in stimulating the prostate and seminal vesicles of a castrated rat than were those implanted subcutaneously. Burrill and Green (29) showed that a testis transplanted into the spleen was not so effective in promoting the growth of the

prostate and seminal vesicles of a castrated rat as was one transplanted subcutaneously. Because the venous drainage of the spleen goes into the portal system, the liver was considered to be the site of inactivation.

Patients with cirrhosis of the liver have been shown by West et al. (24) to excrete only one third as much 17-ketosteroids as normal individuals during testosterone administration. This finding indicates that the liver plays a role in the formation of 17-ketosteroids.

Clark and Kochakian (30)(31) first demonstrated that the liver could metabolize testosterone in vitro. After incubating the hormone with rabbit liver slices, they were able to isolate mostly unchanged testosterone, but some Δ^4-androstene-3,17-dione and small amounts of 17-epitestosterone were present.

In order to detect what products had been formed from the incubation, Clark and Kochakian (31) had to use a greater quantity of testosterone than would dissolve in the incubation medium used; therefore, the question arose whether or not the liver slices would have acted on smaller amounts of the hormone and if the same products would have been produced.

The measurement of the metabolism of testosterone after incubation of quantities which would remain in solution in the aqueous medium was made possible by the development of a method whereby microgram quantities of the steroid could be determined (32). Utilizing the absorption at 240 mμ of the α-β unsaturated ketone in ring A and the Koenig reaction (33), the amount of

destruction of testosterone could be detected, and by an adaptation of the Zimmermann (34) reaction the formation of a 17-ketone could be determined.

By the application of this method, Samuels et al. (1) were able to show that liver from the rat, mouse, rabbit, and human was able to destroy the α-β unsaturated ketonic structure of testosterone while rabbit uterus, rat prostate and seminal vesicles, and mouse mammary tissue were inactive. The reaction could be inhibited by boiling, or by cyanide, iodoacetate, flouride, malonate, or an atmosphere of nitrogen.

Later work by Samuels et al. (35) indicated that livers of birds and mammals destroyed the α-β unsaturation of ring A of testosterone and also produced 17-ketosteroids, while livers from fish, amphibians, and reptiles destroyed the α-β unsaturation at a slow rate but did not form 17-ketosteroids. Dog kidney seemed to be about one-eighth as active as rat liver (36) in destroying the conjugated system of ring A.

Sweat and Samuels (2) then presented evidence that rat liver catabolized testosterone by two different routes. One required DPN and formed 17-ketosteroids while the other was activated by citrate and did not form 17-ketosteroids. Later, Sweat et al. (37) were able to purify partially an enzyme from liver that formed Δ^4-androstene-3,17-dione from testosterone. The change that takes place in ring A of testosterone during incubation with liver tissue remained to be determined.

II. Metabolism of Methyltestosterone:

During the same year in which they had synthesized testosterone, Ruzicka et al. (38) prepared 17α-methyltestosterone and found it to be androgenically active. Soon after its preparation this synthetic steroid was reported to be more effective orally than testosterone in rats (39) and humans (40). The reason for the greater comparative activity of methyltestosterone when administered by mouth is not yet known. However, some differences have been noted in the metabolism of the two steroids.

No increase in androgenic material or in 17-ketosteroids has been found in the urine of animals receiving methyltestosterone (41)(42)(43), while the urine of those receiving testosterone contains greater quantities of both androgenic material and 17-ketosteroids. Even though the difference in metabolism of the two steroids has been apparent for some time, the chemical nature of the compounds excreted when methyltestosterone is catabolized has not been determined.

Again the liver seems to be a site for the catabolism of this steroid. Biskind (44), using the same implanting technique with which he studied testosterone, found methyltestosterone to be inactivated when it was absorbed into the portal system. Burrill and Greene (45) then compared the inactivation of methyltestosterone with testosterone when the two steroids were implanted into the spleens of animals. These authors came to the conclusion that the liver had a greater capacity to inactivate testosterone than methyltestosterone.

In view of the inactivation that takes place in the liver, Hyde et al. (46) thought that the difference between the oral effectiveness of the steroids might be due to the absorption of methyltestosterone through the lacteals, thereby bypassing the liver. To test their proposal, they administered 17α-C^{14}-methyltestosterone intragastricaly to rats and measured the amount of radioactivity entering the thoracic duct. Only 1.5 per cent entered by this route. It appears, therefore, that methyltestosterone is absorbed primarily into the portal vein and that the difference in oral effectiveness between the androgens is not due to a different route of absorption.

Levedahl and Samuels (3) have compared the metabolism of testosterone and methyltestosterone by incubating them with liver slices from dog, rat, chicken, guinea pig, monkey, rabbit, and human. The conjugated system of ring A of both steroids was destroyed by all the species listed, while 17-ketosteroids were produced only from testosterone. When DPN was added to the incubation mixture, with testosterone as substrate, an increased formation of 17-ketosteroids as well as an increased destruction of the α-β conjugated system of ring A was noted. No activation of the destruction of methyltestosterone was observed in the presence of the co-enzyme. However, the addition of citrate to the incubation medium caused an increased destruction of the α-β unsaturation of both steroids.

III. Metabolism of the A Ring of Progesterone:

Venning and Browne (47) were the first to show that increased amounts of pregnanediol glucuronide appeared in the urine of women after they had been given progesterone. Since then, men (48), hysterectomized women (49)(50), and rabbits (51)(52) have been shown to excrete pregnane-3α,20α-diol after treatment with progesterone. In addition to pregnanediol, small amounts of pregnane-3α-ol-20-one from a diabetic man (53) and allopregnane-3α,20α-diol from women (54) have been isolated following progesterone administration.

Like testosterone, only a small amount of the administered dose of progesterone has been recovered. Sommerville and Marrian (55), using intramuscular injections of progesterone in oil found that they could recover only 9 to 16 per cent as pregnanediol. A slightly better recovery was obtained by Gallagher (56) when he administered progesterone-4-C^{14} to women. He was able to isolate 30 per cent of the radioactivity in the urine.

The destruction of progesterone in the body of an animal was first observed by Zondek (57). He found that 48 hours after injection no progesterone could be detected in an extract of the whole body of the animal when decidual formation in the uterus of immature rabbits was used as the assay, but he did not determine where the destruction took place.

By pellet implantation (58)(59) and by injection of progesterone directly into the portal vein (60), the liver has been

implicated as a site for the inactivation of this hormone.

In vitro experiments have also demonstrated that liver tissue is capable of catabolizing progesterone. Samuels (36) reported that the conjugated system of ring A of progesterone was destroyed when it was incubated with rat liver mince. Later, Wiswell and Samuels (61) confirmed this finding with broken cell preparations. The destruction could be accelerated by the addition of citrate, isocitrate, cis-aconitate, cysteine, and cyanide, but not by DPN. They also observed no inhibition when a nitrogen atmosphere was used during the incubation. Because of the activation shown when metal binding agents are present, it was concluded that the enzyme system is inhibited by metal ions.

Taylor (62) was able to confirm the findings of Wiswell and Samuels (61) except that he reported an activation of destruction on the addition of DPN. He also isolated allopregnane-3,20-dione and allopregnane-3α-ol-20-one as metabolites of the incubation.

By incubating large quantities of progesterone with rat liver slices, Horwitt and Segaloff (63) were able to isolate and identify pregnane-3α,20α-diol.

Later, Taylor (64) incubated a series of flasks containing microgram quantities of progesterone with rabbit liver homogenates and then isolated the steroids from the pooled extracts of the incubation mixture. Pregnane-3α,20α-diol and pregnane-3α-ol-20-one were present in the greatest concentration, then came

unchanged progesterone followed by allopregnane-3α-ol-20-one, allopregnane-3,20-dione, and allopregnane-3β-ol-20-one.

IV. Metabolism of the A Ring of the Corticoids:

Cuyler et al. (65) first reported that pregnane-3α,20α-diol was excreted in increased amounts in the urine after the administration of 11-desoxycorticosterone to a normal man. However, they were unable to confirm their original findings when both men and women were given desoxycorticosterone (66)(67). Later, Horwitt et al. (68) found increased amounts of pregnanediol in the urine of 3 men and 1 woman receiving desoxycorticosterone therapy. Rabbits and chimpanzees (69) have also been shown to excrete increased quantities of pregnanediol in their urine after they had been given desoxycorticosterone.

The major end product of 11-dehydrocorticosterone metabolism in man was found by Mason (70) to be pregnane-3α,20α-diol-11-one.

After giving corticosterone to a patient with rheumatoid arthritis, Engel et al. (71) were able to isolate allopregnane-3α,11β,21-triol-20-one, pregnane-3α,11β,21-triol-20-one, pregnane-3α,21-diol-11,20-dione, and pregnane-3α,20α-diol-11-one from the urine.

Both cortisone acetate and cortisol acetate were given to men by Burstein et al. (72)(73) and the excretion products determined. From both steroids the major metabolites were pregnane-3α,17α,21-triol-11,20-dione and etiocholane-3α,11β-diol-17-one. Pregnane-3α,11β,17α,21-tetrol-20-one and

etiocholane-3α-ol-11,17-dione, as well as cortisol and cortisone, were also present in increased amounts.

When the guinea pig was given cortisol, no products were isolated which had been reduced in ring A, although the 20-ketone had been reduced and a 6-β-hydroxyl group had been added (74).

It appears that ring A of all the corticoids studied is reduced during their metabolism in man and all other mammals tested except the guinea pig. Whether or not the guinea pig is unique in its treatment of cortisol will be resolved only by further investigation.

Using the same method by which they implicated the liver in the catabolism of testosterone and methyltestosterone, Burrill and Greene (75) showed that pellets of desoxycorticosterone were inactivated when they were implanted intramesenterically.

Nelson and Harding (76) injected cortisone into dogs and then determined the 17-hydroxysteroids in arterial and venous blood samples taken from various places in the body. Marked differences were shown between the arterial and heptic venous samples. No significant arterio-venous differences were found when samples were taken across other tissues. It, therefore, seems that the liver is playing a major role in metabolizing the corticoids.

In vitro perfusions of rat livers with both cortisone and cortisol by Capsi et al. (77)(78) have indicated that the liver is capable of catabolizing these steroids to a variety of

products. From the perfusate when cortisone was used, allopregnane-3α,17α,21-triol-11,20-dione, allopregnane-3β,17α,21-triol-11,20-dione, allopregnane-3α,11β,17α,21-tetrol-20-one, Δ^4-pregnene-11β,17α,21-triol-3,20-dione, Δ^4-pregnene-17α-20,21-triol-3,11-dione and androsterone were isolated. All five of the products found from the perfusion with cortisol-4-C^{14} were different from those formed from cortisone. They were: allopregnane-3α,11β,17α,20α,21-pentol, Allopregnane-3α,11β,17α,21-tetrol-20-one, Δ^4-pregnene-11β,17α,20α,21-tetrol-3-one, androstane-3α,11β-diol-17-one, and Δ^4-androstene-11β-ol-3,17-dione.

Schneider and Horstmann (79) have incubated desoxycorticosterone with rat liver slices and mince. In 3 hours 35 per cent of the α-β unsaturation of ring A and 25 per cent of the side chain were destroyed. The enzyme system was active under a nitrogen gas phase and was not activated by citrate. Kidney slices were about one third as effective as liver slices in destroying the conjugated system of ring A but fully as active in degrading the side chain. Diaphragm segments were about one fourth as active in destroying both ends of the molecule while adrenal halves did not degrade the side chain but showed some destruction of the conjugated system. Later Schneider (80) identified allopregnane-3α,21-diol-20-one, allopregnane-3β,21-diol-20-one, allopregnane-21-ol-3,20-dione, and allopregnane-3α-20,21-triol as products of the incubation.

These same workers (81) then incubated 11-dehydrocorti-

costerone, cortisone, cortisol, and 11-desoxy-17-hydroxy-corticosterone with liver slices. A destruction of both the side chain and the α-β unsaturated system of ring A was noted in all of the steroids. The best substrate used was 11-dehydro-corticosterone while the worst was 11-desoxy-17-hydroxycorti-costerone. When cortisone was incubated with different tissues, liver slices were found to be more active than kidney slices, diaphragm segments, adrenal homogenate, heart slices, or rectus abdominus muscle mince.

Recently Forchielli et al. (82) incubated a supernatant, prepared from rat liver by centrifuging a homogenate at 600 x g. for 30 minutes, with 11-desoxy-17-hydroxycorticosterone. The products of the incubation were found to be allopregnane-3α,17α, 21-triol-20-one, allopregnane-3β,17α,21-triol-20-one, allopreg-nane-17α,21-diol-3,20-dione, and Δ^4-pregnene-17α,20β,21-triol-3-one.

The most extensive study of the systems in liver which catabolize ring A was done by Tomkins and Isselbacher (83)(84) (85), with cortisone and cortisol as substrates. After ultra-centrifugation of a rat liver homogenate, the supernatant would catalyze the formation of pregnane-3α,17α,21-triol-11,20-dione from cortisone. Further purification of the enzyme system by ammonium sulfate fractionation and calcium phosphate gel adsorp-tion permitted the identification of the first step of the reaction sequence as the saturation of the double bond at posi-tion 4. This reduction is a specific one in regard to both the

cofactor required and the product formed. Only TPNH is active, and only the 5β configuration is obtained. With further purification of the enzyme, they found that only cortisone was reduced. They concluded that different enzymes catalyze the reduction of the double bond in ring A of cortisone, cortisol, and adrenosterone.

Tomkins (86) also obtained a purified enzyme from liver that catalyzed the reduction of the ketone at position 3. Both C_{19} and C_{21} steroids as well as those with the 5β and 5α configuration were reduced; however, the 5β compounds were much better substrates. The carbonyl group at C_{17} was not changed and neither was the 3 carbonyl of the C_{27} steroids. In addition, the 3 hydroxyl formed always had the α configuration.

IN SUMMARY, it appears that the Δ^4-3-ketone of ring A, which is present in all of the nonbenzenoid steroid hormones, is preferentially reduced before the steroid skeleton is excreted from the bodies of mammals. There may be mammals which do not catabolize ring A of some of the hormones; thus far the guinea pig receiving cortisol is the one exception noted. Of all the tissues investigated, the liver contains the most active system for the destruction of the α-β unsaturation of ring A.

A comparison of the products excreted after testosterone administration with those produced from the hormone by the liver cannot be made at the present time. Since the compounds formed when the conjugated system of ring A is destroyed have not been identified, one can only speculate whether these steroids have

the same stereo-configuration as those excreted.

The synthetic androgen 17α-methyltestosterone is also inactivated by the liver and it appears to be catabolized by the same system which destroys the α-β unsaturation of testosterone.

When the excreted metabolite of progesterone is compared to those formed during incubation with liver, a close parallel is found. Pregnane-3α,20α-diol is the major product of each transformation. At least in the case of progesterone, the liver is capable of forming the excreted end product of its metabolism.

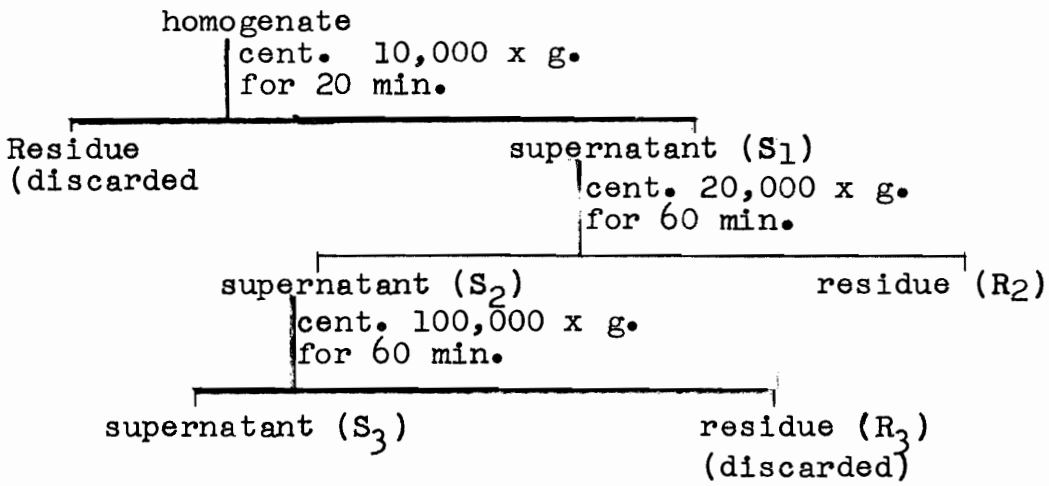
An apparent discrepancy is found when the products arising from metabolism of the corticoids in vivo and in vitro are compared. All of the compounds isolated from the in vivo experiments, excepting one, had the pregnane nucleus. In both perfusions and incubations where large amounts of steroids were incubated with liver preparations, the only products isolated that had a reduced ring A contained the allopregnane nucleus. A different picture is obtained, however, when the amount of steroid incubated resembles more closely physiological concentrations, and a purified enzyme preparation is used. When cortisone is incubated under these conditions, only the pregnane nucleus is isolated and this is in agreement with the products excreted from a normal man given the same steroid.

EXPERIMENTAL METHODS

I. Tissue Preparation:

Rat liver was used as the source of enzyme throughout this study. Male albino rats (Sprague-Dawley strain) ranging from 130 to 420 grams were decapitated with a sharp knife. Their livers were quickly excised and placed in a beaker surrounded by crushed ice. The livers were kept chilled until they could be cut into pieces about 1 cm. square just prior to homogenization. After the addition of 25 to 75 ml. of phosphate buffer containing 0.02 to 0.08 molar niacinamide, the liver tissue was disintegrated. During the initial phase of this problem a Ten Broeck (87) hand homogenizer was used. Later, a 'Polytron' high frequency homogenizer, made by Max Wullimann, Selzach, Switzerland, was employed. Approximately 3 minutes were required for complete homogenization, after which enough buffer was added to the homogenate to give a final concentration of 2 ml. of buffer for every gram of tissue. This preparation was then stored at 3° C. until centrifuged.

For forces up to 20,000 x gravity, the homogenate was centrifuged at 0° C. in an International refrigerated centrifuge, model PR-1, with the high speed head No. 269. When 100,000 x g. was required, a Spinco preparative ultracentrifuge model L with a No. 40 head was used. A flow sheet of the centrifugations appears below:



II. Enzyme Fractionation:

1. Ammonium Sulfate Fractionation and Dialysis:

The usual material for this state of purification was either fraction S_2 alone or both fractions S_2 and R_2. Solid ammonium sulfate was added slowly with constant stirring. After the crystals had dissolved, the mixture was allowed to stand for 5 minutes at 5° C., then the precipitate which had formed was sedimented at 20,000 x g. for 10 minutes. To determine the amount of ammonium sulfate needed, the formula derived by Kunitz (88) was used.

The precipitates were dissolved in a minimum amount of buffer and placed in "Visking" cellulose sausage casings which had been presoaked in distilled water for 30 minutes (Manufactured by the Visking Corporation, Chicago, Ill.). Each of the bags was attached to an apparatus which rotated in a beaker containing the dialysis medium. This procedure shortened the dialysis time and conserved buffer. During the dialysis period, which was from 12 to 16 hours, three 4-liter portions of water

or buffer were used. When water was used as the dialysis medium, a precipitate formed. To redissolve the precipitate, buffer was added and the mixture was stirred at room temperature for 5 minutes, then centrifuged for 10 minutes at 20,000 x g. The supernatant solution was then incubated as such or used as starting material for further purification.

2. Calcium Phosphate Gel Adsorption:

Calcium phosphate gel was prepared as described by Keilin and Hartree (89). It was allowed to age three weeks or more before use. In the majority of experiments the solution to which calcium phosphate gel was added was adjusted so that the nitrogen concentration was 3.4 mg. per ml. Then the required amount of the gel (calculated on its dry weight) was added and the mixture thoroughly stirred until an even distribution of the solid was observed. This mixing required about 15 minutes. The mixture was then centrifuged for 10 minutes at 20,000 x g. If further adsorptions were desired, additional quantities of $Ca_3(PO_4)_2$ gel were added to the supernatant solution and the above procedure was repeated. No attempt was made to adjust the nitrogen concentration or volume of the supernatants on subsequent additions of the gel. In order to elute the protein, a volume of 0.1 N phosphate buffer at the desired pH, equal to the volume of the final supernatant obtained, was added to the gel and thoroughly mixed as described above. The suspension was then centrifuged for 10 minutes at 20,000 x g.

3. Ethanolic Fractionation:

To a solution containing the enzyme kept in an ice bath at 0° C., absolute ethanol, kept at a -10° C., was added dropwise. The solution was stirred continuously to avoid a high concentration of ethanol in any one portion. When the desired amount of ethanol had been added, the solution was allowed to remain at -5° to 10° C. for 10 minutes. To collect the precipitate which had formed, the solution was then centrifuged for 10 minutes at 20,000 x g. and -10° C. After centrifugation the supernatant solution was cooled to -10° C., and the cold ethanol was again added until the desired concentration ensued. This solution was let stand, and then centrifuged according to the above procedure. The residues obtained from the centrifugations were quickly dissolved in a minimum amount of cooled phosphate buffer, and stored in the refrigerator at 3° C.

Nitrogen Determination

The nitrogen concentration of samples was determined by a slight modification of the microkjeldahl procedure of Perrin (90).

III. Buffers, Cofactors, and Substrates:

The buffers used in this work are as follows:

Krebs phosphosaline buffer containing 0.04M NaH_2PO_4 + Na_2HPO_4, pH 7.4, 0.0056M KCl, 0.0021M $MgCl_2$, 0.08M NaCl, and 0.001M sodium citrate.

Tris (tris [hydroxymethyl] aminomethane) buffer, 0.04M, pH 7.4 with the same concentrations of

KCl, $MgCl_2$, NaCl, and citrates as the phosphate buffer.

Krebs-Ringer bicarbonate buffer composed of 0.155M NaCl, 0.033M $NaHCO_3$, 0.0061M KCL, 0.0029M $MgSO_4$, 0.0033M $CaCl_2$, 0.0018M KH_2PO_4, having a pH of 7.4.

The following cofactors and substrates were obtained as listed:

Diphosphopyridine nucleotide (DPN). Pabst, 92% pure.

Reduced diphosphopyridine nucleotide (DPNH). Sigma, Purity unknown.

Reduced triphosphopyridine nucleotide (TPNH). Sigma, 90% pure.

17α-methyltestosterone. Ciba

17α-methyltestosterone-20-C^{14}. Ciba, 2 x 10^5 cpm. per μM.

Epitestosterone. Ciba Mp. 216-220°.

Dihydrotestosterone. Ciba Mp. 180-182°.

17α-ethyltestosterone. Ciba Mp. 138.2-139.5°.

Testosterone. Syntex Mp. 155-155.5°.

Testosterone-4-C^{14}. Tracerlab. 1.

Δ^4-androstene-17-amino-3-one. Ciba

Progesterone. Stock solution.

Δ^4-androstene-3-one. Ciba

Δ^4-androstene-3,17-dione. Stock solution.

When DPN, DPNH, and TPNH were used, they were added in

concentrations of 0.4 millimolar. In most of the incubations the cofactor solution was a boiled liver extract. This extract was prepared by boiling small chunks of liver in the phosphate buffer for approximately 10 minutes, homogenizing the mixture, and then centrifuging it at 20,000 x g. for 20 minutes. The supernatant solution was then added to the incubation mixture. Most samples received an amount of extract equivalent to 0.5 gram liver.

The cofactors and substrates were used as they were obtained without further purification.

IV. Incubation Techniques:

The required amount of steroid dissolved in ethanol was added to a 125 ml., glass-stoppered, Erlenmeyer flask. The flask was then placed under an air jet and the ethanol was evaporated. Just before the buffer and cofactor solutions were added to the flask, 0.2 ml. of absolute ethanol was introduced to redissolve the steroid and enhance its solubility in the aqueous solution. Initially the final volume per flask was 25 ml. Later it was changed to 20 ml., and then to 10 ml.

Buffer and cofactor solutions were than added. The enzyme fraction was pipetted into the flask and nitrogen gas or a mixture of 95 per cent oxygen and 5 per cent carbon dioxide was blown over the surface of the liquid. When the O_2-CO_2 mixture was used the flasks were gassed for 5 minutes. In most experiments where nitrogen was used, the gas was bubbled through the buffer for 20 minutes before it was added to the flask. When

this had been done, the flask was gassed for 5 minutes. If it had not been done, 10 minute periods were used.

At the end of the gassing period, the flasks were stoppered and sealed. A mixture formed from heating equal weights of rosin and beeswax together was applied to the top of the ground portion of a heated glass stopper and the stopper was put in the flask while still warm. Ten minutes later the stopper was taped on the top of the flask with adhesive tape and the flask was clamped to a rotating spindle in a constant temperature bath. The flask was then rotated at a desired temperature for the required length of time.

When the incubation period was over, the flask was removed from the bath, unstoppered, and the contents boiled after the addition of 3 drops of octanol, or the stoppered flask was placed in a deep freezer.

V. Steroid Extraction and Purification

The contents of the incubation flasks were transferred to 100 ml. centrifuge tubes after the sealing material had been removed from the ground joint with benzene. Each flask was then rinsed thoroughly with three 6-ml. portions of ethyl acetate and added to the centrifuge tubes. A propeller-tipped stirring rod attached to an electric motor was used to mix the organic and aqueous phases which were then separated by centrifugation. When the separation of the phases was complete, the ethyl acetate was transferred to 125 ml. Erlenmeyer flasks with a "serum lifter". This procedure was repeated twice with 20-ml.

portions of ethyl acetate. The extracts from each sample were then combined and the ethyl acetate was removed by evaporation under a jet of air.

Modified Zaffaroni (91) paper chromatograms were used to purify the ethyl acetate extract. Strips of Whatman No. 2 filter paper were cut to the pattern and dimensions described in Figure 1.

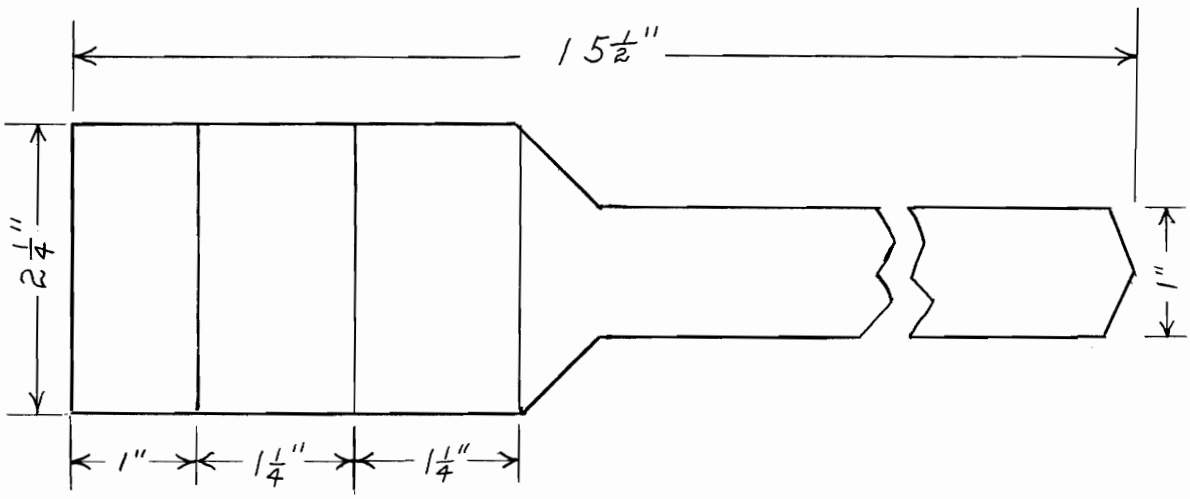


Figure 1 - Pattern of Zaffaroni paper Chromatograms.

The strip was then immersed in a methanol-formamide mixture (1:1), blotted, and hung at room temperature until the methanol had evaporated. At the end of this time, the residue from the ethyl acetate extraction was applied to the origin of the chromatogram and the chromatogram was put in a glass jar saturated with Skellysolve 'C' (Commercial Petroleum distillate composed mainly of heptane).

After a development of 6 hours with Skellysolve C, the paper chromatogram was removed from the tank, dried, and

examined under ultraviolet light to locate steroids having the α-β unsaturation in ring A. This time of development was usually sufficient to elute the fat from the end of the strip. The strip was then placed in another glass jar and developed with dichloroethane overnight. A graduated glass-stoppered test tube was placed below the strip to catch the eluate. Approximately 5 ml. of eluate were collected. The sides of the tube were rinsed with ethanol and the tube was placed under an air jet to increase the evaporation of the solvents.

A small drop of liquid, presumably formamide, remained in the bottom of the tube after the dichloroethane and the ethanol had evaporated. To eliminate this material, 2 ml. of distilled water were added to the tube and the aqueous phase was extracted twice with 4 ml. portions of dichloroethane. The tube containing the extract was then placed under an air jet until the dichloroethane had evaporated. By this procedure, a sample was obtained that gave good symmetrical absorption curves with little interferring background in the ultraviolet spectrum from 2200 Å to 2600 Å.

During the latter part of this work when the incubations contained a purified enzyme fraction and no niacinamide, it was possible to determine the absorption pattern of the ethyl acetate extract without further purification.

The recovery of steroid from a sample boiled immediately after the addition of the enzyme fraction and then carried through the same procedure as the other samples was about 84

per cent with a homogenate and 95 to 104 per cent with all of the other enzyme fractions.

VI. Quantitative Estimations of Steroids.

To determine the amount of α-β unsaturation in a sample, 1.0 ml. of ethanol was added to the residue and the solution was left standing for 1 hour. An aliquot was transferred to a "Cary" recording spectrophotometer cell and diluted with ethanol until the cell held a final volume of 3.2 ml. The ultraviolet absorption pattern of the sample was then read between 2200 Å and 2600 Å against a blank which had been carried through the same steps as the sample from the time of incubation. If any material having α-β unsaturation was present in the sample, a curve with a maximum around 2400 Å appeared. Quantification of such a curve was obtained by the following equations: (92)

(a) $2 \times$ O.D. at 2400 Å $-$ (O.D. at 2300 Å + O.D. at 2500 Å $= \Delta$

(b) $\frac{\Delta \text{ of sample}}{\Delta \text{ of standard}} \times$ quantity of standard $\times$

$$\frac{\text{Total volume of sample}}{\text{Volume of aliquot}} = \text{quantity of material in sample}$$

The ketone group at C-3 was quantitatively determined by the method of Gornall and MacDonald (93). In this method the 2,4-dinitrophenylhydrazone is formed in an acidic methanol solution; then the solution is made basic to enhance and stabilize the color. The brownish red has a maximum absorption at 435 mμ.

Paper partition chromatography was used to separate the

steroids extracted from the incubation medium. The systems used were those described by Bush (94). In his system, the material to be chromatographed is applied to a dry untreated strip of filter paper. The strip is then placed in a jar having an atmosphere saturated with the solvents used to develop the chromatogram, until saturation of the paper is complete. When this situation (equilibration) has been achieved, the developing solvent is added to the trough inside the jar through a hole drilled in the plate glass top and allowed to flow over the paper for the required time.

Sheets of Whatman No. 2 filter paper, 23 inches square, were cut into 7-inch strips, the maximum width which the glass jars used would accommodate. These strips were than divided into a number of lanes as illustrated (Fig. 2). It was found

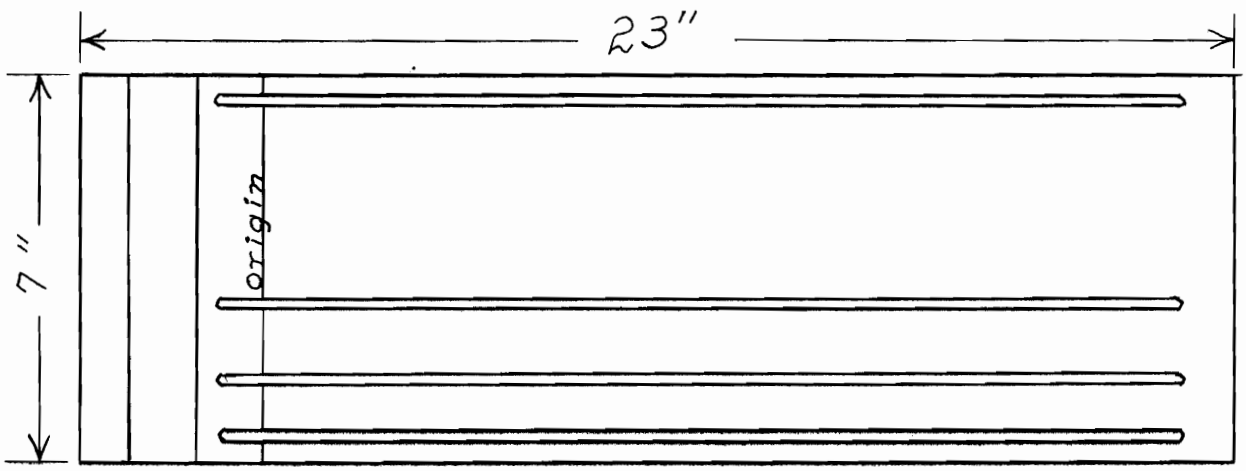


Figure 2 - Pattern of Bush paper Chromatograms.

that 300 micrograms of steroid would move in a compact zone without trailing when applied to a 3-inch lane of paper. Lanes ranging from 1 cm. to 7 inches were used with good results. On the outer lanes at the origin, a small amount of Sudan III dye was applied. This served as a visual gauge of the extent of movement of solvent. When the dye spot was about 6 cm. to 8 cm. from the bottom of the strip, the paper was removed from the jar and dried at 37° C.

The developing solvents used in this work were:

(a) Skellysolve C methanol: water, 85:15 (by volume); (designated hereafter as C-85).

(b) Benzene: Skellysolve C 33:66, saturated with methanol: water, 80:20 (by volume); (designated hereafter as BC-80)

(c) Benzene saturated with methanol: water, 55:45 (by volume); (designated hereafter as B-55)

When the extracts chromatographed were fairly clean and contained very little fat, an equilibration time of 6 hours was sufficient. For cruder extracts an equilibration of from 12 to 16 hours was required. At the end of this time, the solvents were added to the troughs and the chromatogram was allowed to develop.

In order to detect the steroid on the paper, general color reactions, specific color reactions, measurement of radioactivity, and ultraviolet light absorption were used. An ultraviolet light scanner, modeled after the one described by Haines and Drake (95),

was used to locate the steroids having the α-β unsaturation of ring A. The dark areas were encircled with a line drawn by a graphite lead pencil.

When C^{14}-labeled steroids were chromatographed, the radioactive zones were found by cutting a 2 cm. strip from the lane, then dividing this into 2 cm. squares and counting each square in a windowless proportional counter. By knowing the consecutive arrangement of the squares which had formed the strip, the R_f's of the radioactive zones could be calculated. Later, when a strip counter was available, a recorded pattern of the radioactivity on a strip 2 cm. wide was made during the passage of the strip through the counter equipped with a thin window.

An 8 per cent solution of phosphomolybdic acid in absolute ethanol was used to detect Δ^4-unsaturated-3-ones, either Δ^4 or Δ^5 unsaturated diols, and 17α-methylandrostane-3α,17β-diol (Androstane-3α,17β-diol did not react with this reagent). A blue spot appeared where the steroid was located when the paper was sprayed with the reagent and set in a room with a temperature of 37° C. for 15 minutes.

Saturated steroids containing ketone groups were found by applying a saturated solution of 2,4-dinitrophenylhydrazine in 2N aqueous HCl.

A fairly specific reagent for Δ^4 and Δ^5 unsaturated 3-ols was a 15 per cent solution of phosphotungstic acid in absolute ethanol. Of all the compounds tried, the only ones that produced a purplish-red color were those having one of these

configurations. It was also possible to distinguish between Δ^4 and Δ^5 3-ols, since the Δ^5 compounds gave a color containing more red than the Δ^4 compounds.

Three methods were used to elute the steroids from the paper. In the first method, ethanol was drawn from a trough by capillary action onto the paper strip containing the steroid zone and allowed to drip into a container from the slightly pointed end of the paper. In the second method, a paper containing a steroid zone was placed in a Soxhlet apparatus and refluxed for 3 hours with ethanol. The third method consisted of cutting the paper containing the steroid zone into 1 cm. or smaller squares, then letting the squares sit for 15 minutes in a beaker containing 1 ml. ethanol per square centimeter of paper. At the end of this time the ethanol was removed with a pipette. The procedure was repeated twice more, the 3 portions of ethanol were combined and the solvent evaporated under an air jet.

Usually steroids eluted by the first and third methods were sufficiently purified to give a good symmetrical absorption curve in the spectral region from 2200 Å to 2600 Å. In case further purification was desired, the eluted material was dissolved in 5 ml. of dichloroethane and applied to a silica gel column 5 cm. high and approximately 0.7 cm. in diameter. The steroids were then eluted from the column with 0.7 per cent ethanol in dichloroethane.

VII. Preparation of 17α-methylandrostene-3α,17β-diol and 17α-methylandrostene-3β,17β-diol:

To 1.0 gram of methyltestosterone dissolved in a minimum of absolute methanol, 90 mg. of $NaBH_4$ was added with continuous stirring. When the bubbling stopped, distilled water was added to the reaction mixture. The white crystalline precipitate formed was filtered off and washed with water until the filtrate had the same pH as the water. The aqueous filtrate was extracted with three 25-ml. portions of ethyl ether which were combined, and washed with 10 ml. of 0.1N HCl and then with water until neutral. When the ether was evaporated the residue (about 10 mg.) was added to the collected precipitate which had been dried at room temperature.

A solution of 3.23 gm. digitonin in 450 ml. 90 per cent ethanol was prepared, and 250 ml. of this was added to an ethanolic solution of the crystalline reaction products. After standing for two days at room temperature, the digitonide which had precipitated was collected and the volume of the filtrate concentrated to 150 ml. by heating to 45° C. under reduced pressure. To this concentrated filtrate was added the remaining digitonin solution and the mixture was again allowed to remain at room temperature for 2 days, after which time the precipitated digitonide was collected. The combined digitonide fractions weighed 1.0628 grams.

A minimum amount of pyridine was used to dissolve and split the digitonide. The digitonin was precipitated by the addition

of ether and filtered off. The ether solution was washed with dilute HCl and then water, dried over sodium sulfate, and evaporated to dryness. The amount of crude 17α-methyl-Δ^4-androstene-3β,17β-diol obtained was 178 mg.

The volume of the filtrate, remaining from the digitonide collections, was reduced close to dryness, extracted with three 50-ml. portions of ethyl ether, and the extract was filtered. The ether was evaporated to dryness leaving a residue of crude 17α-methyl-Δ^4-androstene-3α,17β-diol, weighing 445 mg.

After 3 recrystallizations from acetone, the melting point of the 17α-methyl-Δ^4-androstene-3α,17β-diol was 170° to 172° C., and that of the 17α-methyl-Δ^4-androstene-3β,17β-diol was 158° to 159.5° C.

VIII. Reactions Used to Identify Steroids:

1. Preparation of Acetates:

Acetylation was used both as a means of determining the presence of unhindered alcohol groups, and for the specific identification of steroid alcohols. Approximately 30 μg. of the material to be acetylated was dried for two days in a vacuum desiccator. One drop of acetic anhydride and one drop of pyridine were then added and thoroughly mixed. When the solution had stood at room temperature for 24 hours, 3 ml. of water were added and the mixture was extracted with two 5-ml. portions of ether. The combined ether extracts were then washed with dilute H_2SO_4, dilute NaOH, and water, dried over Na_2SO_4, filtered and evaporated.

2. Oppenauer Oxidation:

The Oppenauer oxidation was employed to compare the metabolite with the methylandrostenediol and to check for the presence of the Δ^4 or Δ^5 3-ol grouping. Aluminum isopropoxide (8 mg.; Eastman) was added to approximately 75 µg. of the material to be oxidized which was dissolved in 3 ml. of acetone and 5 ml. of benzene. The mixture was then refluxed for 12 hours. To the cooled reaction medium, 2 ml. of 2N HCl were added and the phases were well mixed. The benzene layer was then removed and washed with water, filtered through a small amount of Na_2SO_4, and evaporated to dryness.

3. Preparation of Thiosemicarbazones:

The presence and position of a ketone group was determined by the ultraviolet absorption spectrum of the thiosemicarbazones. Thiosemicarbazones were prepared by a slight modification of the procedure described by Bush (96). To a ground glass-stoppered tube holding approximately 15 µg. of steroid in 0.1 ml. of ethanol was added 0.1 ml. of glacial acetic anhydride containing 5 mg. per ml. of thiosemicarbazide. After the stopper had been taped on the tube, the sample was heated at 100° C. for 40 minutes. After the tube had cooled, the solution was made up to 0.3 ml. with ethanol, and transferred to a microspectrophotometric cell. The absorption spectrum from 200 mµ. to 320 mµ. was then recorded.

4. Radioactive Counting Techniques:

The radioactive measurements were accomplished by counting

aliquots of solutions and paper strips containing the radioactive compound. Material in solution was plated on aluminum discs. During the process of plating, the disc was mounted on an electrically driven turntable and rotated while a stream of air was directed on it to enhance the evaporation of the solvent. In order to control the solution flow so that an equal distribution of the radioactive material was obtained, a fine-tipped pipette attached to a small syringe was used.

Most of the plating was done with ethanol as the solvent. When amounts of crystalline material of 3 mg. or more were plated on each disc, a more even distribution was achieved by using 70 per cent aqueous methanol. Both the aluminum discs and the paper squares previously referred to were counted with a gas flow counter.

5. Recrystallization to Constant Specific Activity:

To determine if the radioactive material was the same as the carrier employed, the mixture was recrystallized a number of times to see if a constant specific activity was attained. A portion of the radioactive material was added to a compound with which it was assumed to be identical. The mixture was then recrystallized, the radioactivity of the crystals determined, and the specific activity (CPM/mg.) calculated. After a second recrystallization and calculation of the specific activity, the first and second specific activities were compared. If they were different, then more recrystallizations were performed until the specific activity between two consecutive recrystalli-

zations was the same or until it was apparent that a constant specific activity would not be achieved. When two consecutive recrystallizations produced a constant specific activity, it was assumed that the crystallization pattern and structure of both compounds were the same; therefore, one criterion of identity had been satisfied, and the carrier was considered to be the same as the radioactive component. When a constant specific activity was not obtained, the recrystallization pattern of the two compounds was assumed to be different; therefore, the carrier was not considered to be the same as the radioactive component.

6. Comparative Solubility Technique:

The identicalness of a radioactive component and the compound used as a carrier was checked by comparative solubilities. A procedure patterned after that described by Gutman and Wood (97) was used. Approximately 20 ml. of material which had been recrystallized to a constant specific activity was divided into two portions. The smaller portion contained approximately one third of the material. This portion was then incubated at a constant temperature in a volume of solvent in which it was not completely soluble, until equilibrium between solid and solution was obtained. An equilibrium was assumed when aliquots taken consecutively at 24-hour intervals showed the same amount of radioactivity per unit of solution. When this was attained, the remaining two-thirds of the crystalline material was added to the solution. The mixture was again incubated until the same criterion of equilibrium had been achieved. If the radioactive

material and the carrier were the same compound and true equilibrium had been reached in each case, the radioactivity per unit volume of the solution should have been the same after the addition of the whole sample as after the addition of one third of the sample. If the two were different, the solution would not be expected to be saturated with the radioactive compound and more should dissolve.

EXPERIMENTAL RESULTS

I. Attempts to Identify Product From the Incubation of Methyltestosterone:

The first step in determining the route by which methyltestosterone was metabolized was the identification of the first major product which accumulated in incubations with rat liver homogenate. One micromole of methyltestosterone was incubated for 1 hour at 36.5° C. with the quantity of homogenate prepared from 0.5 gm. of rat liver. When the extract of the incubation medium was chromatographed in the C-85 system, a spot was found moving behind methyltestosterone. The metabolite was detected by spraying the chromatogram with phosphomolybdic acid. No evidence of α-β unsaturated was found upon examination of this area on the ultraviolet scanner. This spot was radioactive when 17α-methyltestosterone-20-C^{14} was incubated, indicating it to be a metabolite of methyltestosterone.

In order to find some evidence of the identity of this metabolite, all of the available 17-methylated steroids were chromatographed in the C-85 system and the R_f values compared with that of the metabolite. Table I lists the R_f values of these compounds. From an examination of the table, it is obvious that the R_f of the metabolite did not agree with that of any of the known 17-methyl steroids that were chromatographed.

TABLE I

COMPARISON OF THE R_f VALUES OF 17-METHYLATED STEROIDS IN THE BUSH C-85 SYSTEM

STEROID	R_f
17α-methyltestosterone	0.32
17α-methyl-Δ^5-androstene-3α,17β-diol	0.18
17α-methylandrostane-3α,17β-diol	0.32
Metabolite	0.22

Approximately 20 μg. of steroid were applied to the origin of a strip 18 mm. wide. The strips were placed in tanks containing the C-85 system and equilibrated for 6 hours. They were then developed for approximately 4 1/2 hours. Spots were located by examination on an ultraviolet scanner and by spraying with phosphomolybdic acid.

It is also apparent that the R_f values for methyltestosterone and 17α-methylandrostane-3α,17β-diol were the same within the limits of error of the measurement. To determine if there was a difference in the chromatographic behavior of these compounds which was too small to measure after such a short distance of movement, the paper strips were developed for extended time periods. Approximately 20 hours were required for the separation of the two compounds.

Since the metabolite moved more slowly than methyltestosterone and faster than 17α-methyl-Δ^5-androstene-3α,17β-diol, the assumption was made that it was more polar than methyltestosterone

and less polar than the 17α-methyl-Δ^5-androstenediol. Because the movement was closer to the enediol than the anediol, it was possible that it could be one of the 17α-methyl-Δ^4-androstenediols. These compounds were not available commercially so they were synthesized. Both the 3α and the 3β isomers of 17α-methyl-Δ^4-androstenediol had the same R_f value. Both gave a positive color reaction with phosphomolybdic acid.

A comparison of the chromatographic movement of the metabolite and the 17α-methyl-Δ^4-androstenediols showed them to be similar in this respect. The R_f values of the 17α-methyl-Δ^4-androstene-3α,17β-diol and the metabolite in BC-80 system were 0.39 and 0.41, respectively. When a mixture of radioactive metabolite and the diols was made and chromatographed, only one spot was observed. The blue color obtained with phosphomolybdic acid and the radioactivity were found in the same area. Since a difference of 0.02 in R_f values may be obtained with the same compound chromatographed in adjacent lanes, and because only one spot was noted in the lane containing the mixture, it appeared that one of the diols and the metabolite might be identical.

To make a further comparison, the acetates of the metabolite and the 3α and 3β isomers of the methyl-Δ^4-androstenediol were compared and chromatographed in the C-85 system. The following R_f values were obtained:

Metabolite	0.87
17α-methyl-Δ^4-androstene-3α,17β-diol	0.85
17α-methyl-Δ^4-androstene-3β,17β-diol	0.85

The movements of the free compounds and the acetates indicated that the metabolite might be one of the methyl-Δ^4-androstenediols. Whether the metabolite had the 3α or the 3β configuration could not be determined by chromatography in the two Bush systems since the movement of these two isomers was identical. In order to distinguish between these compounds, some of the radioactive metabolite was added to each of the diols and the specific activities of the crystals determined after a series of recrystallizations (Table II). A constant specific activity appeared to be attained after the second recrystallization when 17α-methyl-Δ^4-androstene-3α,17β-diol was the carrier while the specific activity of the 17α-methyl-Δ^4-androstene-3β,17β-diol was still decreasing after the third recrystallization.

From the evidence presented, it appeared that the metabolite was 17α-methyl-Δ^4-androstene-3α,17β-diol. To test this conclusion, the constant solubility experiment described under Experimental Methods was performed. One third of a sample containing the mixture of radioactive metabolite and 17α-methyl-Δ^4-androstene-3α,17β-diol that had been recrystallized to constant specific activity was placed in a tube containing just enough 70-per cent methanol to dissolve all but a few crystals. The tube was placed vertically in a constant temperature bath with a shaking mechanism and incubated at 38° to 40° C. Aliquots of the solution were then plated and counted at 24-hour intervals until constant radioactivity was obtained. When this

TABLE II

RECRYSTALLIZATION OF THE RADIOACTIVE METABOLITE

COMPOUND USED AS CARRIER	CPM/mg.
17α-methyl-Δ^4-androstene-3α,17β-diol	
2d recrystallization	349
3d recrystallization	374
17α-methyl-Δ^4-androstene-3β,17β-diol	
2d recrystallization	338
3d recrystallization	226

Radioactive metabolite was added to 17 mg. of 17α-methyl-Δ^4-androstene-3β,17β-diol and to 15 mg. of 17α-methyl-Δ^4-androstene-3α,17β-diol. Both compounds were recrystallized from warm acetone. Aliquots of the recrystallized material were plated from 70-per cent methanol and counted.

had been achieved the remaining two-thirds of the sample was added to the tube and it was again incubated until the solution showed a constant radioactivity. The results of this experiment are shown in Table III. Since the radioactivity of the solution increased when a greater amount of compound was present, either the radioactive product was not the same as the carrier or equilibrium had not been achieved originally. In Experiment No. 2 the tube was placed in the bath horizontally rather than vertically to increase the mixing and to enhance better equilibration. The results obtained, however, were the same as those from Experiment No. 1. It seemed unlikely that a similar degree of failure to achieve equilibrium would have occurred in both experiments.

TABLE III

CONSTANT SOLUBILITY OF RADIOACTIVE METABOLITE

	CPM/ml.	
	Exp. 1	Exp. 2
Addition of 1/3 of sample		
1st aliquot	675	540
2d aliquot	645	517
Addition of remaining 2/3 of sample		
aliquot	914	826

Experiment No. 1: 6.9 mg. of the radioactive metabolite and 17α-methyl-Δ^4-androstene-3α,17β-diol which had been recrystallized to a constant specific activity was added to 2 ml. of 70-per cent aqueous methanol. First aliquot was taken 24 hours after addition and second aliquot 48 hours after. The remaining 13.5 mg. were then added. Last aliquot was taken 48 hours after the addition of the larger portion.

Experiment No. 2: 5.6 mg. was added to 2 ml. 70-per cent methanol. Aliquots taken 24 and 48 hours after addition. 11.3 mg. were then added. Final aliquot taken 48 hours after last addition.

The identity of the two compounds, therefore, was open to question in spite of their similar chromatographic behavior and their recrystallization to constant specific activity in one solvent system. To gain further evidence about the identicalness of the steroids, the absorption patterns of the sulfuric acid chromogens of both compounds were determined by the method of Zaffaroni (91). The metabolite gave two peaks with maxima at 210 mμ and 387.5 mμ while the methylandrostenediol gave two

peaks with maxima at 310.5 mμ and 408 mμ 1 hour after the addition of the acid. When the samples were analyzed 2 hours and 4 hours after the acid had been added, a change was found in the optical densities but the maxima of the curves had not changed. The slight differences observed in the absorption spectra were not considered great enough to indicate whether or not the compounds were identical.

An Oppenauer oxidation was then carried out on both compounds, and the reaction products were chromatographed in the BC-80 system. Figure III shows the results obtained. The oxidation of the methylandrostenediol produces a compound that absorbs light in the ultraviolet region and moves the same distance as methyltestosterone, while the product isolated from the oxidation mixture of the metabolite moves the same distance and shows the same characteristics as the unchanged metabolite. Since the Oppenauer oxidation of Δ^4-3-ols and Δ^5-3-ols has been shown to produce the Δ^4-3 ketone in ring A (98), it appears that the metabolite contained neither of these configurations.

Since it appeared that the two compounds were different, a further attempt was made to separate them chromatographically. A mixture of the metabolite and 17α-methyl-Δ^4-androstene-3α,17β-diol was chromatographed in the C-85 system; a developing period of 13 hours was used rather than the normal of 4 1/2 hours. Figure IV illustrates that a separation of the compounds was possible when this increased time of development was employed.

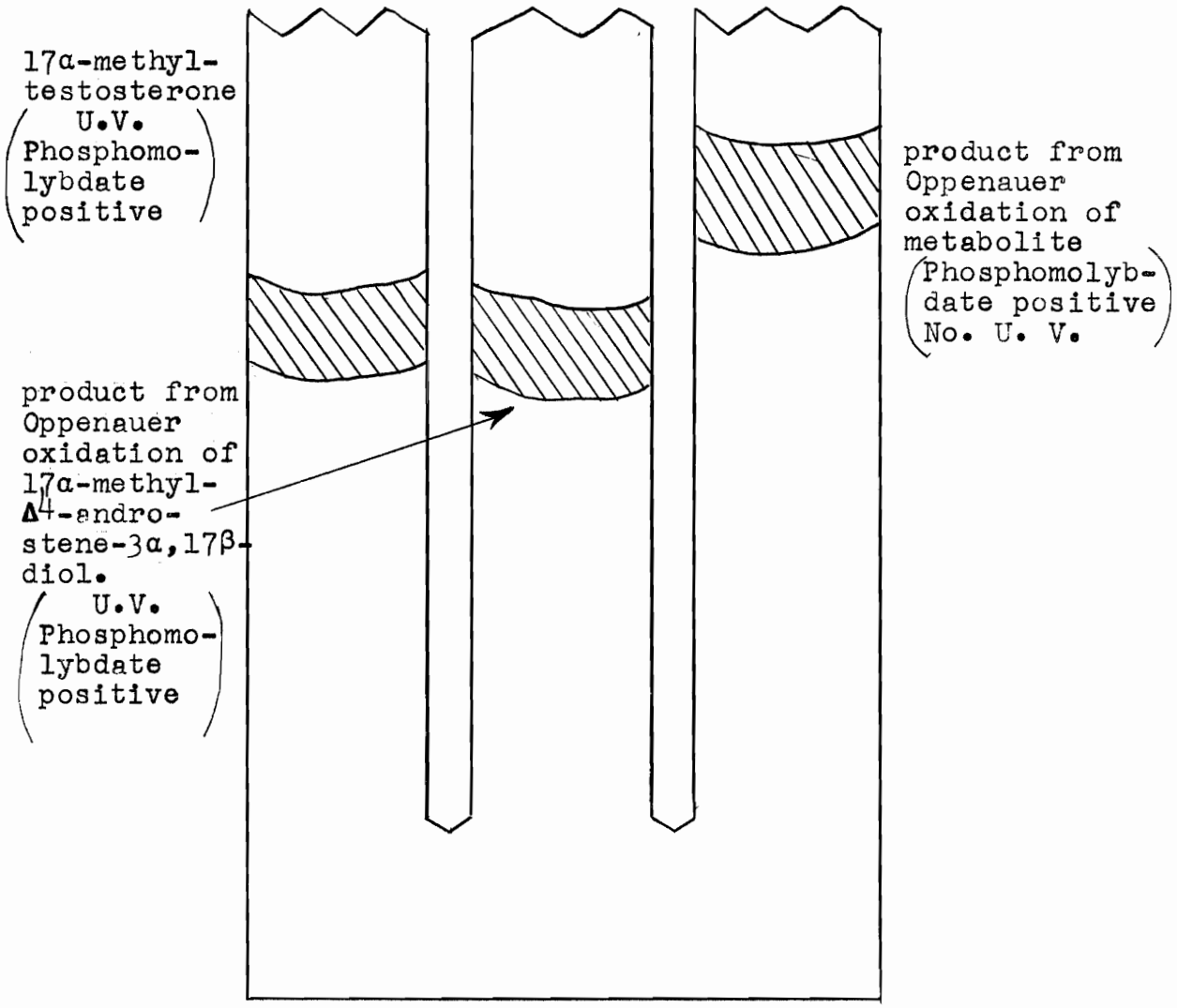


Figure 3 - Chromatographic pattern of products of Oppenauer oxidation.

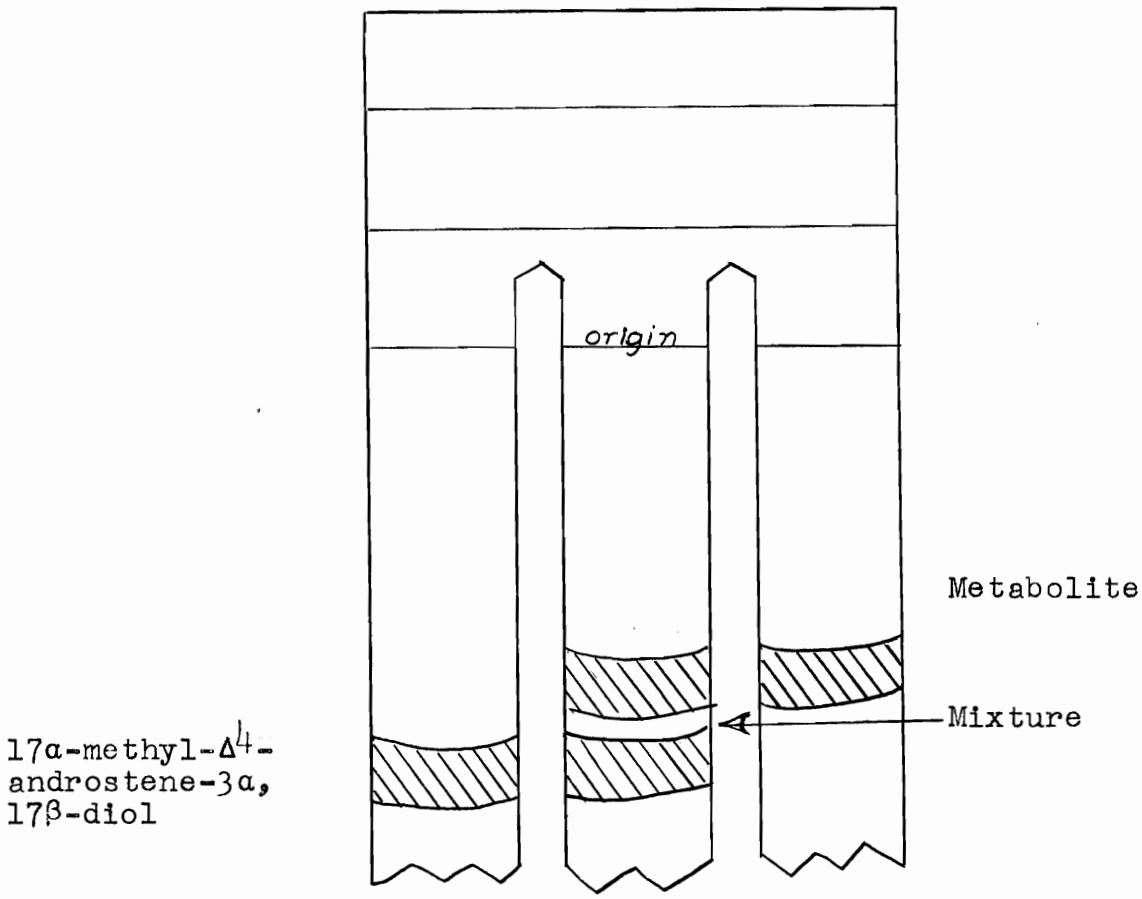


Figure 4 - Chromatographic separation of metabolite and 17α-methyl-Δ^4-androstene-3α,17β-diol.

From the evidence obtained it was fairly well established that the metabolite was not 17α-methyl-Δ^4-androstene-3α,17β-diol even though the free compounds and acetates of both steroids chromatographed alike and the radioactive metabolite recrystallized to a constant specific activity with the diol as the carrier when one solvent system was used.

The possible presence of a ketone group in the metabolite was checked with 2,4-dinitrophenylhydrazine and with thiosemicarbazide. When the hydrazine was added to a paper strip

containing a spot of the metabolite, no deeper yellow spot appeared. This finding indicated that a hydrazone had not been formed. When a reaction mixture containing thiosemicarbazide and the metabolite was subjected to ultraviolet absorption analysis, no curve having a maximum in the spectral region from 260 mμ to 350 mμ was obtained. Two compounds, dihydrotestosterone and androstane-3-one, having ketone groups at C_3, formed substances in the presence of thiosemicarbazide which absorbed ultraviolet light. Dihydrotestosterone produced a curve with a maximum at 318 mμ and androstane-3-one produced a curve with a maximum at 320 mμ. Bush (96) claims that the curves given by thiosemicarbazones of saturated 17-ketones show a maximum at 269 to 271 mμ. Because no absorption curve having a maximum in either of the regions indicated was obtained with the metabolite, it was concluded that the metabolite does not contain a ketone group at C_3 or C_{17}. Furthermore, since neither a 2,4-dinitrophenylhydrazone nor a thiosemicarbazone could be detected, it was concluded that the metabolite contained no ketone groups.

Supporting evidence for the absence of a Δ^4 or a Δ^5 grouping in the metabolite was obtained by spraying paper strips containing the metabolite with phosphotungstic acid. The purple-red color characteristic of these configurations did not appear. Instead, a dirty tan closely resembling that given by 17α-methyl-androstane-3α,17β-diol appeared. Evidence for or against a Δ^1 grouping was not obtained.

The metabolite appeared to be still a C_{20} steroid. When 200,000 cpm. of 17α-methyltestosterone-20-C^{14} were incubated with liver homogenate, 164,000 cpm., or 82 per cent was recovered. This recovery is in agreement with the 167,000 cpm. recovered from a sample containing 200,000 cpm. of methyltestosterone which had been boiled immediately after the addition of the enzyme fraction. Because the recovery of the added radioactivity was in the same range, whether or not enzymatic action had occurred, it appears that the methyl group at C_{20} was not split off during the metabolism of methyltestosterone.

The information accumulated about the metabolite then indicates that it does not contain a ketone group or a Δ^4 or Δ^5 grouping. In all probability it has a saturated ring A, but it is not 17α-methylandrostane-3α,17β-diol because the two compounds move differently when they are chromatographed. It could be the 3α isomer of methylandrostanediol, but since the 3α and 3β isomers of methylandrostanediol did not behave differently during chromatography there is no reason to believe the anediols would. The most probable assumption is that the metabolite is one of the methyletiocholanediols.

In addition to the major metabolite of methyltestosterone, which has just been discussed, at least two other radioactive areas were found. One of these was more polar than the major metabolite and less polar than corticosterone. When this substance was chromatographed on a more narrow lane, at least two compounds of very similar polarity appeared to be present.

These areas did not appear to absorb ultraviolet light when examined on the scanner.

The other radioactive area had a polarity between that of corticosterone and that of cortisone. Because only one compact spot was detected on the chromatogram by analysis and by the color produced with phosphomolybdic acid, this radioactive compound appeared to be chromatographically pure. An examination of it on the scanner showed it to absorb ultraviolet light.

Table IV is a comparison of the R_f values of these metabolites with corticosterone, cortisol, and cortisone.

TABLE IV

COMPARISON OF SOME CORTICOIDS WITH METABOLITES OF 17α-METHYLTESTOSTERONE-20-C^{14}

COMPOUND	R_f
Metabolite (absorbing U. V. light)	0.58
Corticosterone	0.69
Cortisol	0.252
Cortisone	0.40
Metabolite (no U. V. light absorption)	0.84

Approximately 20 μg. of each steroid was chromatographed. Cortisone and cortisol were put on the same 18 mm. lane. The paper strip was equilibrated for 16 hours and developed for 4 hours in the B-55 system. All R_f values were converted from R_d values obtained. The metabolites were located by their radioactivity.

Since the primary objective was the investigation of the major product of methyltestosterone metabolism in which the α-β unsaturation of ring A was destroyed, and the enzyme system responsible for this destruction, the other metabolites were not investigated further.

IN SUMMARY, it appeared that the first major product accumulating in the metabolism of methyltestosterone by rat liver was a non-ketone alcohol with a saturated ring A. In view of the two groups on the molecule that need to be reduced in order for this condition to be achieved, it was probable that more than one enzyme was required in the conversion of methyltestosterone to the metabolite. The object of this investigation then became a determination of whether or not more than one enzyme was involved.

II. Cofactor Requirements:

The enzyme catalyzing the formation of the metabolite from methyltestosterone was obtained in the soluble state. An active, clear, cherry-red solution was obtained when a homogenate of rat liver was centrifuged at 20,000 x g. and the supernatant was filtered through Celite. Table V, Experiment No. 1, shows that the supernatant was slightly more active than the homogenate.

In order to determine if the enzyme was present in the minute particles of the cell, the supernatant from the centrifugation at 20,000 x g. was recentrifuged at 100,000 x g. The results (Table V, Experiment No. 2) indicate that all of the activity was in the soluble fraction.

TABLE V

METABOLISM OF 17α-METHYLTESTOSTERONE BY RAT LIVER FRACTIONS

FRACTION	Steroid Reduced μM/gm. liver/hr.
Experiment No. 1*	
Original homogenate	0.64
Clear supernatant from 20,000 x g. Cent.	0.81
Experiment No. 2*	
Residue from the 100,000 x g. Cent.	0
Supernatant from 100,000 x g. Cent.	0.86

*One micromole of methyltestosterone was incubated per flask with the enzyme fraction for 1 hour at 36.5° C. The incubation medium contained Krebs phosphosaline buffer, pH 7.4+DPN. Each flask contained a final volume of 25 ml. An O_2-CO_2 gas phase was used.

When the homogenate was prepared in the absence of niacinamide, the supernatant was completely inactive. An active supernatant could also be inhibited considerably by dialyzing it against a large volume (12 liters) of phosphate buffer. The loss of either niacinamide or DPN was not responsible for this inhibition, because when they were added back, alone or in combination, to the dialyzed medium they did not restore the activity. In one experiment, a combination of metal ions, including 0.001M $FeCl_2$, 0.001M $FeCl_3$, 0.001M $ZnCl_2$, 0.001M $MnCl_2$, and 0.001M $CuCl_2$, failed to restore activity.

It was then found that a boiled extract of liver would restore the activity of the dialyzed enzyme fraction. Two preparations of boiled liver were compared. Fresh rat livers were cut into small squares and divided into two equal portions to each of which buffer was added. One portion was boiled and filtered to give a clear yellow filtrate while the other portion was boiled and homogenized. The results in Table VI indicate that the required cofactor or cofactors are not tightly bound to the liver tissue since the filtrate was as active as the whole liver preparation. When the liver was homogenized before it was boiled, the extract was inactive, and activity was not restored when niacinamide was added immediately after boiling.

In view of the protection of the cofactors by niacinamide during homogenization, it was probable that the cofactor was one of the phosphopyridine nucleotides. DPN had already been shown to be inactive. In one experiment and with one incubation flask, TPN was also inactive. When DPNH was incubated, it was

TABLE VI

COMPARISON OF THE BOILED LIVER FRACTIONS

FRACTION	Steroid Reduced μM/gm. liver/hr.
Supernatant	0.53
Dialyzed supernatant	0.21
Dialyzed supernatant + boiled filtrate	0.57
Dialyzed supernatant + boiled homogenate	0.53

One micromole of methyltestosterone was incubated per flask with the enzyme fraction for 1 hour at 36.5° C. The incubation medium contained Krebs phosphosaline buffer, pH 7.4+DPN. Each flask contained a final volume of 25 ml. An O_2-CO_2 gas phase was used.

found to be completely ineffective in restoring the activity in the presence of either an oxygen or nitrogen gas phase. It was then found that the activity of the enzyme could be restored by TPNH during incubation in a nitrogen gas phase. Later experiments have shown that the enzyme is as active in the presence of NaH_2PO_4 + Na_2HPO_4 pH 7.4 + TPNH as it is in the presence of the Krebs phosphosaline buffer, described under Experimental Techniques, and boiled liver filtrate. An examination of the products of the incubation with TPNH as cofactor showed a compound appearing which had the same characteristics as the major metabolite previously described. There were no compounds containing ketone groups detectable with 2,4-dinitrophenylhydrazine, or Δ^4 or Δ^5-3-ols detectable with phosphotungstic acid.

Therefore, TPNH was found to be the only cofactor required for the metabolism of ring A of methyltestosterone to a saturated alcohol. There was no evidence of significant accumulation of ketonic or unsaturated intermediates in this system.

III. Enzyme Fractionation:

1. First Ammonium Sulfate Fractionation:

The supernatant from the centrifugation at 20,000 x g. was fractionated with ammonium sulfate. An amount of ammonium sulfate necessary to produce the desired concentration was added to the supernatant. After the precipitate which had formed was collected by centrifugation, it was dissolved in buffer and dialyzed against buffer or water. The dialyzed enzyme fraction was then incubated with methyltestosterone. Table VII, Experiment No. 1, shows that the majority of activity was precipitated when the ammonium sulfate concentration was 60 per cent of saturation but not when it was 30 per cent of saturation.

Further fractionation revealed that most of the activity was precipitated at 50 per cent of saturation but not at 40 per cent of saturation (Table VII, Experiment No. 2).

The product of incubation of methyltestosterone with this purified enzyme fraction appeared to be the same as that isolated from the homogenate incubations. It moved the same distance when it was chromatographed and gave a positive color reaction with phosphomolybdic acid. No evidence was found to indicate the presence of a ketone when the extract was

TABLE VII

AMMONIUM SULFATE FRACTIONATION OF THE ENZYME

FRACTION	Steroid Reduced μM/hr.
Experiment No. 1	
Supernatant	0.42
30%$(NH_4)_2SO_4$ ppt.	0.056
60%$(NH_4)_2SO_4$ ppt.	0.87
100%$(NH_4)_2SO_4$ ppt.	0.089
Remaining Supernatant	0.043
Experiment No. 2	
40%$(NH_4)_2SO_4$ ppt.	0.22
50%$(NH_4)_2SO_4$ ppt.	0.71
60%$(NH_4)_2SO_4$ ppt.	0.11

One micromole of methyltestosterone was incubated per flask with the enzyme fraction for 1 hour at 36.5° C. The incubation medium contained Krebs phosphosaline buffer, pH 7.4+DPN. Each flask contained a final volume of 25 ml. An O_2-CO_2 gas phase was used.

chromatographed and the strip sprayed with 2,4-dinitrophenylhydrazine. There were also no detectable Δ^4 or Δ^5-3-ols when the strip was sprayed with phosphotungstic acid.

2. Calcium Phosphate Gel Fractionation:

To a dialyzed solution of the material precipitating at 50 per cent ammonium sulfate saturation, 25 mg. of calcium phosphate gel were added per ml. After the gel was blended with the solution, it was collected by centrifugation. To the supernatant 50 mg. of the gel were then added per ml. and the blending and centrifugation were repeated. The protein that had been

adsorbed on the gel was eluted with 0.1M phosphate buffer, pH 7.4. The amount of purification and the units of enzyme present in the fractions are presented in Table VIII.

Elution of the enzyme adsorbed on the calcium phosphate gel was carried out at different pH's. Table VIII lists the pH's used and the activity of the enzyme obtained. Since the best activity was obtained at pH 7.4 this was the pH that was used for elution in the remainder of the experiments.

TABLE VIII

CALCIUM PHOSPHATE GEL FRACTIONATION

FRACTION	Steroid Reduced μM/mg. N_2	Total Activity* μg.reduced/hr.
Super. (20,000 x g.)	0.016-0.026	9600
40-50% $(NH_4)_2SO_4$ ppt.	0.056	4000
25 mg. $Ca_3(PO_4)_2$ gel/ml. super.	0.11	1900
25 mg. $Ca_3(PO_4)_2$ gel/ml. eluate (pH 7.4)	0.069	
50 mg. $Ca_3(PO_4)_2$ gel/ml. super.	0.10	
50 mg. $Ca_3(PO_4)_2$ gel/ml. eluate (pH 6.5)	0	
50 mg. $Ca_3(PO_4)_2$ gel/ml. eluate (pH 7.0)	0.059	
50 mg. $Ca_3(PO_4)_2$ gel/ml. eluate (pH 7.4)	0.12-0.14	600

*Total Activity = $\frac{\text{Total volume of enzyme solution}}{\text{Volume of solution required to reduce 1 μg. steroid.}}$

During this fractionation, the purification of the enzyme was 5 to 8-fold above that of the supernatant. However, the same metabolite that was formed by the ammonium sulfate fraction and the homogenate was produced, and no intermediates were found.

3. Ethanolic Fractionation:

The supernatant obtained from the adsorption on 25 mg. per ml. calcium phosphate gel was fractionated with cold ethanol. The precipitates that formed when the ethanolic concentrations were 20 per cent, 30 per cent, 40 per cent, and 50 per cent by volume were collected and incubated. In Table IX it can be seen that the greatest purification was found in the material precipitated when 40 per cent ethanol was present.

TABLE IX

ETHANOLIC FRACTIONATION

FRACTION	Steroid Reduced μM/mg. N_2	Total Activity* μg. reduced/hr.
25 mg. $Ca_3(PO_4)_2$ gel/ml. super.	0.11	1900
Per cent Ethanol:		
20	0.036 †	
30	0.046 †	
40	0.159	400
50	0	

*Total Activity = $\frac{\text{Total volume of enzyme solution}}{\text{Volume of solution required to reduce 1 μg. steroid.}}$

†These values were estimated from the amount of phosphomolybdic acid color reduced.

When the products of the incubation with the fraction precipitated by 40 per cent ethanol were chromatographed, a spot which reacted with phosphomolybdic acid was found moving at the same rate as the major product from the homogenate incubation. No ketones were detected with 2,4-dinitrophenylhydrazine, and no Δ^4 or Δ^5-3-ols were found with phosphotungstic acid.

4. Second Ammonium Sulfate Fractionation:

The material eluted from the precipitate after centrifugation of the 50 mg. per ml. calcium phosphate gel addition was fractionated with ammonium sulfate. Table X illustrates the fractions taken and the purification obtained. The only active portion was that precipitating at 60 per cent saturation. A small amount of purification resulted from this procedure and it is the most consistant one found so far.

Here again no ketones or Δ^4 or Δ^5-3-ols were detected in the extract from the incubation medium. The product that was isolated had the same characteristics as that produced with the other fractions.

During the fractionation of the liver preparation by the procedures just described, a certain amount of purification resulted. However, neither a sharp increase in activity occurred, nor was there any indication that more than one enzyme was present. The same product was formed by all the enzyme fractions prepared and at the end of the incubation there was an absence of any detectable intermediates.

TABLE X

SECOND AMMONIUM SULFATE FRACTIONATION

FRACTION	Steroid Reduced μM/mg. N_2	Total Activity* μg. reduced/hr.
50 mg. $Ca_3(PO_4)_2$ gel/ml. eluate (pH 7.4)	0.12-0.14	600
ppt. from Per cent $(NH_4)SO_4$ Saturation		
20	0	
40	0	
60	0.182	300
80	0	
100	0	

*Total Activity = $\frac{\text{Total volume of enzyme solution}}{\text{Volume of solution required to reduce 1 μg. steroid.}}$

IV. Attempts To Trap An Intermediate:

Another approach intended to indicate whether the reduction of ring A involved more than one stage and required more than one enzyme was attempted. It was postulated that by incubating a possible intermediate with radioactive testosterone which had been shown to be a substrate for the enzyme system (see Table XIII), the radioactivity would be trapped in the compound if the substance did indeed serve as product of one and substrate of a second enzyme. Four micromoles of dihydrotestosterone (DHT) were incubated in the presence of testosterone-

4-C^{14} having a specific activity of 16,000 cpm per micromole. The specific activities of the major metabolite formed, and of the DHT isolated were then calculated and compared. From the results in Table XI it is apparent that the specific activity of the metabolite was about 10 times that of the DHT. Since there was some activity trapped in the DHT, the reduction must have taken place in at least two steps. The low specific activity of the DHT, however, would indicate that either it was not an intermediate on the main metabolic path, or that it was never completely free in the medium.

TABLE XI

SPECIFIC ACTIVITY OF INTERMEDIATE

COMPOUND	μM	CPM	Specific Activity CPM/μM
Metabolite	2.7	8812	3255
DHT	1.5	533	355

A crude separation of the steroids from the incubation mixture was made on a silica gel column. The fractions obtained were then chromatographed in the C-85 system and the steroids separated and eluted. Quantitative estimation of DHT was made using the method of Gornall and MacDonald. The amount of metabolite was calculated from the amount of testosterone and DHT destroyed.

V. Effect of Different Buffers:

To determine if phosphate ions were inhibiting the enzyme system, an incubation using phosphosaline, tris, and bicarbonate buffers was performed. The enzyme was active in both the Krebs

phosphosaline and Tris buffers. However, very little activity was shown in the Krebs-Ringer bicarbonate buffer. The μM of steroid destroyed per gm. of liver per hour for each of the buffers are listed below:

Krebs phosphosaline	0.32
Tris	0.44
Krebs-Ringer bicarbonate	0.079

Because the bicarbonate buffer contained calcium ions and sulfate ions which were not present in the other buffers, it was postulated that either one or both of these ions might be inhibitory. When calcium ions were added to an incubation medium containing phosphate buffer, an inhibition was not observed (Table XII). The presence of versene during an incubation neither inhibited nor increased the enzymatic activity. No inhibition was detected when sulfate ions were added to the incubation. Therefore, the cause of the inhibition in the bicarbonate buffer remains undetermined. The values obtained also suggest that the activity of the enzyme may be greater in the Tris buffer than in the phosphate buffer. If any inhibition is caused by the phosphate ions, it is slight.

VI. Inactivation of Enzyme:

The enzyme was found to be fairly labile to standing, heat, and pH change. After standing in the refrigerator at 3° C. for two days, it had lost about 50 per cent of its activity. There did not seem to be any change in the rate of inactivation between the crude and more purified enzyme fractions.

When the enzyme was preincubated in the presence of air or oxygen for one hour at 36.5° C. it was completely destroyed. An atmosphere of nitrogen would preserve 81 per cent of the activity under the same conditions. The enzyme was also destroyed when it was heated in air to 60° C. for 4 minutes.

The enzyme lost about 50 per cent of its activity when it was allowed to stand for 10 minutes at a pH of 5.8 and a temperature of 3° C.

VII. Inhibitors:

A number of inhibitors which could contribute some information about the site involved were incubated with methyltestosterone (Table XII). Cyanide was included to determine if the activity were dependent on the transfer of electrons to a porphyrin-bound iron ion. If iron ions were necessary and the activity depended on the oxidation and reduction of these ions, azide would inhibit by keeping the iron reduced to the ferrous state. The importance of sulfhydryl groups was determined by the effect of p-chloromercuribenzoate. Calcium ions were included because of the inhibition found with the bicarbonate buffer, and the requirement for metals was determined with versene.

It is apparent that the sulfhydryl group is required for activity. In view of this it is rather difficult to explain the inhibition of the cyanide since this ion has been shown to activate enzymes requiring the sulfhydryl groups. The only explanation that can be offered at this time is that the high

TABLE XII

INHIBITION OF ENZYME

INHIBITOR	Steroid Reduced μM/hr.	% Inhibition
$NaHN_3$ (0.001M)	0.6	0
KCN (0.01M)	0.55	50
PCMB* (0.001M)	0	100
Ca^{++} (0.08M)	0.52	0
Versene (0.001M)	0.52	0

*p-chloromercuribenzoate

TPNH was used as the cofactor, and N_2 as the gas phase. Otherwise, the conditions of incubation were the same as those listed under Table V. The enzyme fraction used was the 40 to 50 per cent ammonium sulfate cut from the supernatant of a 100,000 x g. centrifugation.

concentration used was, in some way, inhibitory. Because versene did not inhibit the enzyme it seems that no metal ions are required for activity.

In view of the inhibition of the enzyme by p-chloromercuribenzoate, it was thought that cysteine might protect the enzymatic activity. Neither increased activity nor protection of the enzyme was found when it was present in a concentration of 0.005M.

VIII. Effect of Citrate:

Citrate had been shown to activate the enzyme system that destroyed the α-β unsaturation of ring A of both testosterone and methyltestosterone when they were incubated with liver slices

and homogenates (2)(3). In these more purified enzyme fractions an activation by citrate was not observed.

IX. Substrate Specificity:

Table XIII shows the relative rates of reduction of a number of steroids containing the Δ^4-3-ketone grouping in ring A. It is apparent that the compound having the more polar group on the 17-position is the better substrate. The stereo-configuration of this group does not change the affinity of the enzyme for the substrate appreciably since the molecule having the 17α-hydroxyl group is almost as active as testosterone. The atomic constituents of this group do not decrease its substrate activity appreciably, as is shown by the comparative activities of the compounds having the 17-amino group and the 17-hydroxyl group. Although a substituted polar group on C-17 increases the activity of the substrate it is not necessary since the compound having only hydrogen atoms at this position is still 60 per cent as active as one having maximum activity. Increasing the number of carbons on position 17 appears to decrease the steroid's effectiveness as a substrate. 17α-methyltestosterone was less active than testosterone but more active than progesterone or Δ^4-pregnene-17β-ol-3-one.

X. Effect of Substrate Concentration:

The dependence of enzymatic activity on substrate concentration when an O_2-CO_2 gas phase was present is shown in Figure 5. An enzyme concentration comparable to that used in the other

TABLE XIII

SUBSTRATE SPECIFICITY

SUBSTRATE	Steroid Reduced μM/hr.	Relative Rate of Reduction
Testosterone	0.69	100
Epitestosterone	0.66	96
Δ^4-androstene-17-amino-3-one	0.65	94
Δ^4-androstene-3,17-dione	0.56	81
17α-methyltestosterone	0.55	80
Progesterone	0.44	64
Δ^4-pregnene-17β-ol-3-one	0.42	62
Δ^4-androstene-3-one	0.40	60

The amount of each steroid incubated was approximately 1 μM per flask. A final volume of 20 ml. was incubated in each flask; otherwise the conditions of incubation were the same as those listed under Table V.

experiments was employed. At this enzyme concentration the Michaelis constant is approximately 1.65×10^{-5} M.

When it was discovered that the enzyme was destroyed in an O_2-CO_2 gas phase, the incubation was again performed with N_2 (Figure 6). Under these conditions the rate is faster for a concentration of 2 micromoles than for 1. It also seems that a concentration of 3 micromoles per 20 ml. inhibits the enzyme. If the straight line portion of the graph is extrapolated to infinite substrate concentration a K_m of 2.64×10^{-5} is calculated (99).

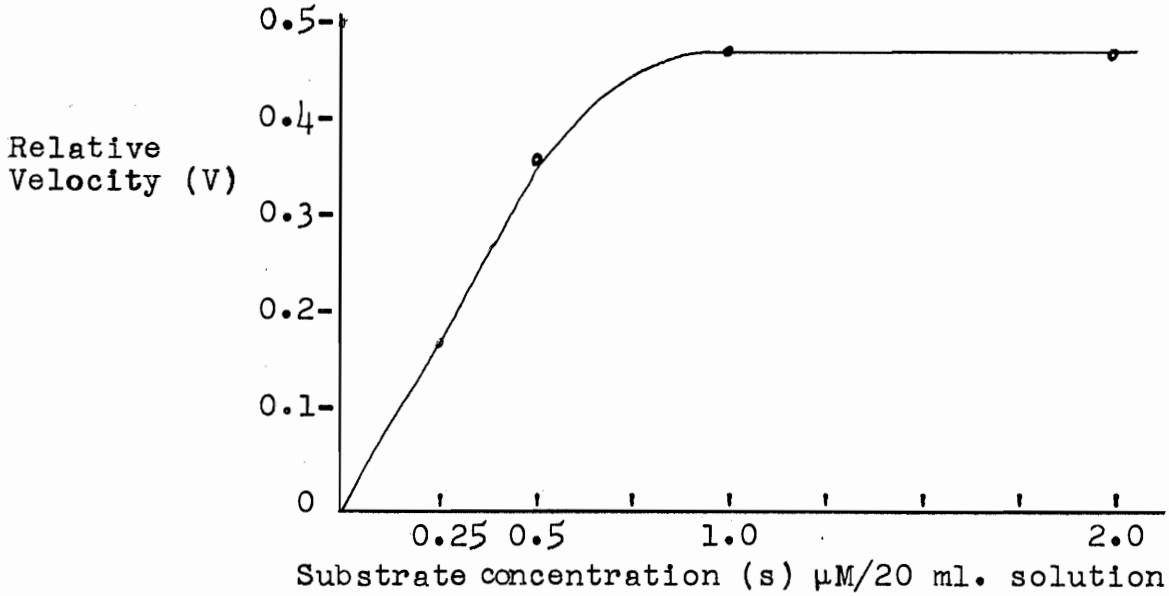


Figure 5 - Plot of relative velocity against substrate concentration. Flasks were incubated for 1 hour at 36.5° C. with an O_2-CO_2 gas phase. The enzyme fraction used was 40 to 50 per cent ammonium sulfate cut.

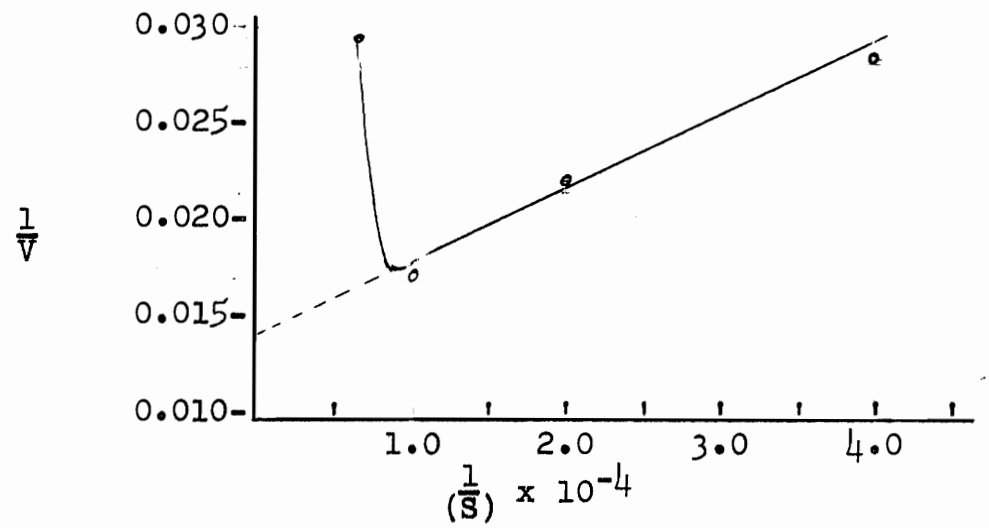


Figure 6 - Plot of the reciprocal velocity against the reciprocal substrate concentration. The flasks were incubated for 1 hour at 36.5° C. with a nitrogen gas phase. The enzyme fraction used was a 40 to 50 per cent ammonium sulfate cut.

XI. Effect of Length of Incubation:

When the enzyme was incubated with methyltestosterone in the presence of an oxygen-carbon dioxide gas phase, the amount of the α-β unsaturation destroyed was the same after 1 hour as it was after 3 hours. It was then observed by preincubation for 1 hour that the enzyme was completely destroyed in an O_2-CO_2 gas phase. Therefore, the apparent equilibrium which had been reached after 1 hour was due to the destruction of the enzyme. When nitrogen, however, was used as the gas phase during incubation, an equilibrium was not reached at the end of 3 hours (Figure 7). It can also be seen that the rate of reaction was slightly faster during the initial phase of the incubation, but no sharp change occurred as the incubation progressed.

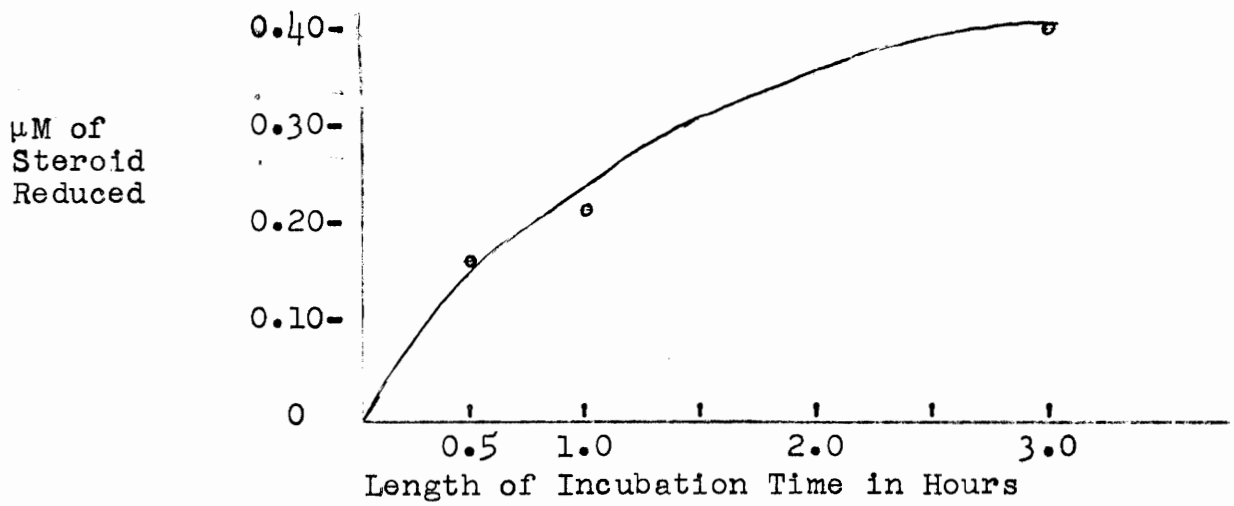


Figure 7 - μM of steroid reduced as a function of time. Methyltestosterone was incubated at 36.5° C. in phosphosaline buffer pH 7.4 under a nitrogen gas phase.

XII. Effect of Incubation Temperature:

From an examination of Figure 8, it is apparent that two temperature optima have been found for the enzyme system destroying the α-β unsaturation of ring A of methyltestosterone. One optimum appears at about 36.5° C. while the other optimum appears at approximately 50° C.

An examination and comparison of the products of incubation formed from the 36.5°, 45°, and 50° C. incubations disclosed no difference in the products formed. No ketones were detected with 2,4-dinitrophenylhydrazine and no unsaturated 3-ols were detected with phosphotungstic acid. The amount of major product also appeared present in a quantity directly comparable to the amount of α-β unsaturation destroyed.

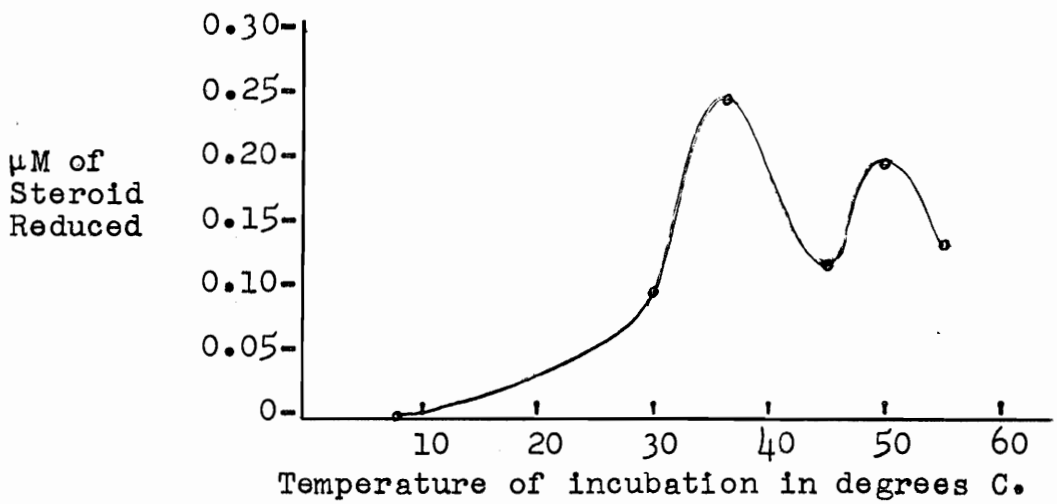


Figure 8 - μM of methyltestosterone reduced as a function of temperature. One μM was incubated in a volume of 10 ml. of phosphate buffer. The enzyme fraction used was a 40 to 50 per cent ammonium sulfate cut. A nitrogen atmosphere was used and the flasks were not rotated during incubation. The two optima were obtained in two separate experiments.

XIII. Effect of pH:

The effect of pH from 6.5 to 8.5 on the rate of the reaction was determined. Figure 9 shows a curve that appears to have two optima; one at 7.4 and another at 6.5 or lower. Almost the same rate of reaction is found at pH 6.5 as that at 7.4.

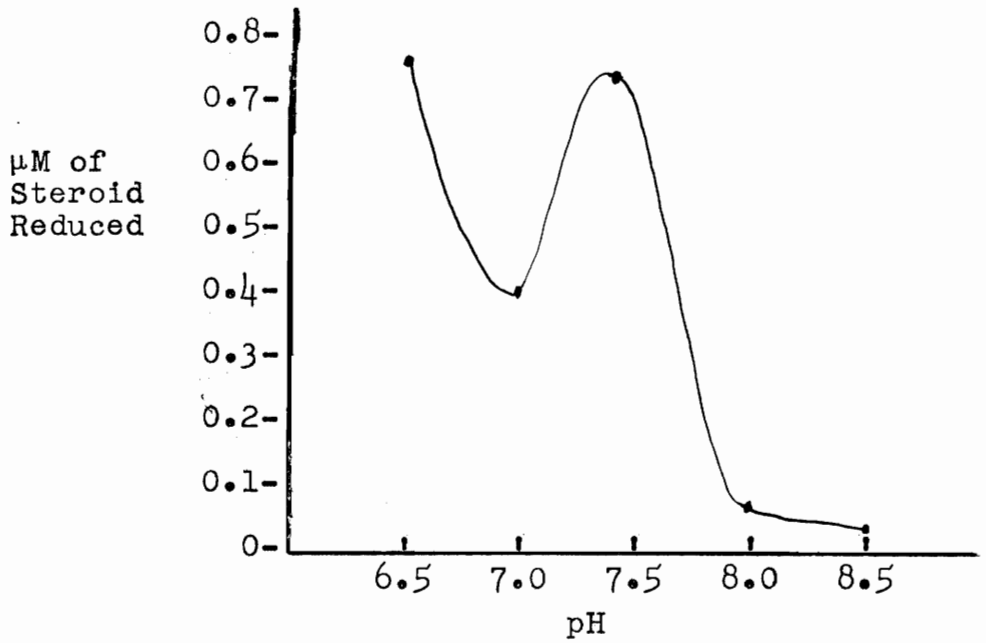


Figure 9 - μM of methyltestosterone reduced as a function of pH. The incubation was performed at 36.5° C. for 1 hour.

XIV. Effect of Enzyme Concentration:

Figure 10 shows the dependence of the rate of reaction on enzyme concentration. It is surprising that no activity was found with 0.5 ml. of enzyme solution. Only one experiment of this type was performed. It then remains a question as to whether or not the absence of activity at this low concentration can be repeated. If this low value is discarded, a fairly good fit of a straight line that runs through the origin can be

obtained with the other values.

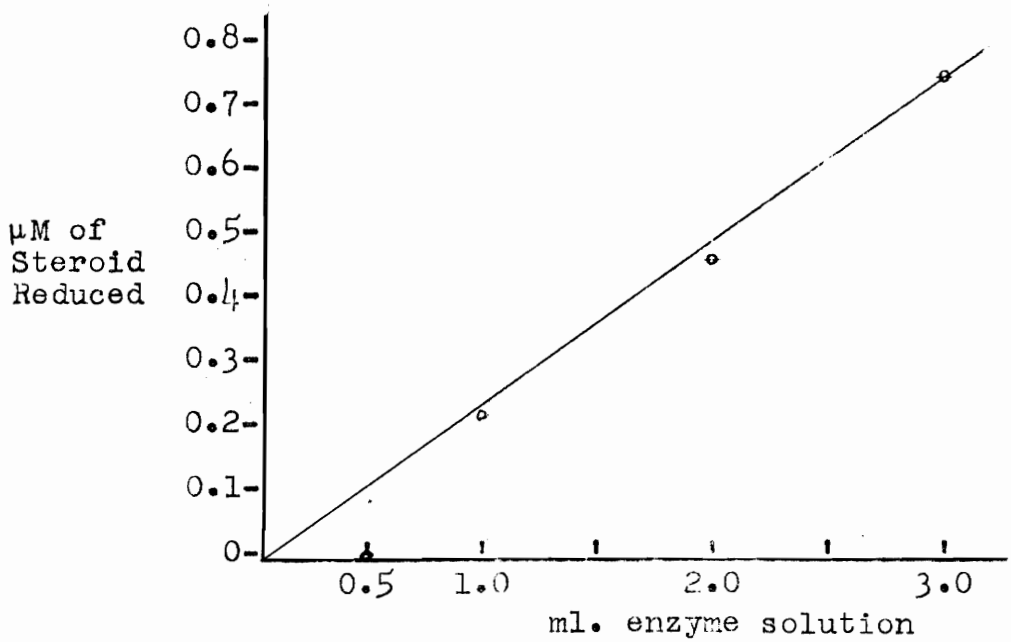


Figure 10 - μM of methyltestosterone reduced as a function of enzyme concentration. The incubation was performed at 36.5° C. for 1 hour under a nitrogen gas phase. The enzyme fraction used was 40 to 50 per cent ammonium sulfate cut.

XV. Incubation in the Presence of Tritiated Water:

In order to see if the hydrogen ions that were transferred to the double bond and the 3 ketone group were donated by the aqueous solution, tritiated water was added to the incubation medium. 17α-methyltestosterone and the major metabolite were incubated with TPNH in a phosphosaline buffer containing 1 mc. of tritiated water to which no enzyme had been added. This was done to determine if there were any exchange of the hydrogen atoms of the steroid and the solution. Less than 2 cpm. per micromole was found in both compounds. It is, therefore,

apparent that there is a negligable amount of exchange between the hydrogen atoms of the steroid and those of the aqueous medium. When the metabolite which had been formed in the presence of tritiated water was analyzed it contained 1494 disintegrations per minute. Calculation of the activity in the metabolite formed in the presence of tritium appears below:

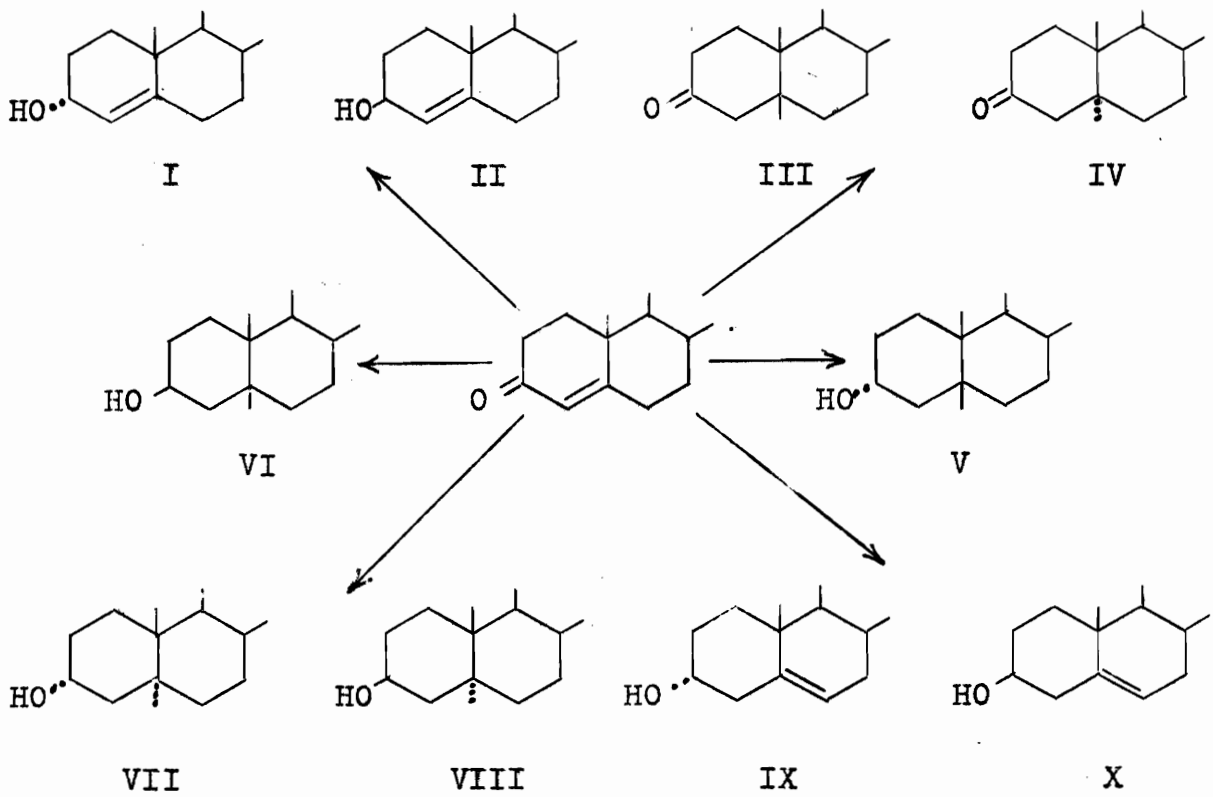
At a pH of 7.4, there would be a hydrogen ion concentration of 4×10^{-6} moles per liter of water. For a volume of 10 ml. there would be 4.0×10^{-8} moles of H^+. The amount of tritiated water added was 0.05 ml. or 5×10^{-3} of the total amount present, therefore, $4.0 \times 10^{-8} \times 5 \times 10^{-3} = 2.0 \times 10^{-10}$ of H^+ contributed by the tritiated water. Since the tritiated water is composed of THO then $\frac{2 \times 10^{-10}}{2} = 1 \times 10^{-10}$ moles of T^+ present in the incubation medium. One milicurie of tritium was present in 0.05 ml. of 2.8×10^{-3} moles of water, therefore, the specific activity is $\frac{2.2 \times 10^{9}}{2.8 \times 10^{-3}} = 7.85 \times 10^{11}$ disintegrations per minute per mole of tritium. Since 1 micromole of 17α-methyltestosterone had been incubated and 67 per cent of it had been reduced then 2.7×10^{-6} moles of H^+ had been transferred to the steroid. The amount of tritium that would have been expected to be transferred was then $\frac{1 \times 10^{-10}}{4 \times 10^{-8}} \times 2.7 \times 10^{-6} = 6.75 \times 10^{-9}$ moles. Because the specific activity of the tritium was calculated to be 7.58×10^{11} disintegrations per

minute per mole and 6.75×10^{-9} moles could be expected to transfer if all of the hydrogen ions were donated by the medium, the metabolite should contain 5.1×10^{3} disintegrations per minute. It appears that approximately 29 per cent of the hydrogen used for reduction is donated from the medium.

DISCUSSION

I. Structure of the Metabolite Isolated:

If one considers the possibilities of the structure of the major metabolite, assuming that the only change of methyltestosterone during the incubation is a reduction of ring A, the possible structures appear below:



The assumption that the only major change during metabolism is a reduction of ring A is not unlikely. Levedahl's (100) attempt to trap radioactivity in $BaCO_3$ when 17α-methyltestosterone-20-C^{14} was incubated with rat liver mince was unsuccessful. This finding has been confirmed in the present work because the same percentage of recovery of radioactivity was obtained from samples containing active enzyme as from those that had been boiled immediately after the addition of the enzyme. Since there is essentially no loss of radioactivity during the metabolism, it is evident that the carbon at the 20 position is not lost. It is conceivable that there could be a rupture of one or more of the rings during metabolism. This, however, seems improbable because the metabolite moves in the Bush chromatography systems at about the same rate as the intact 17α-methylated steroids. If an opening of a ring had taken place, the material formed would not be expected to move like a steroid. Therefore, the evidence is consistent with the assumption that the only change that takes place in the major metabolite when methyltestosterone is incubated is a reduction of ring A.

A consideration of structures I and II has been made in this investigation. The compound represented by structure I was differentiated from the metabolite, after a series of indecisive experiments, by an Oppenauer oxidation and a final chromatographic separation during an extended period of development. The failure of the compound represented by structure II to recrystallize to a constant specific activity

with the radioactive metabolite eliminates it as the possible structure. It then appears that the metabolite does not have the configuration of either structure I or II, even though both the free compounds and their acetates behaved in a manner chromatographically similar to the metabolite and its acetate.

The compounds represented by structures III and IV have also been shown to be different from the metabolite. Neither a thiosemicarbazone nor a dinitrophenylhydrazone has been formed by the metabolite. With the concentrations of the metabolite used, a ketone group should have reacted with the reagent. Another steroid postulated on the metabolite, DHT, reacted when present in an equivalent concentration. There is no reason to believe that steric hinderance could explain the negative reactions of the metabolite provided it contained a ketone group. In addition, since a saturated compound having a ketone should be less polar than an unsaturated ketone, the metabolite, if it had a saturated ketone, would be expected to move between methyltestosterone and the front. This was in contrast to the results obtained because the metabolite moved between methyltestosterone and the origin. Therefore, the probability that the metabolite has either structure III or IV or even a ketone group on the molecule is rather small.

The compound having structure VII behaves differently from the metabolite when the two are chromatographed in the same system. The metabolite, therefore, does not have structure VII.

A more tentative argument is used to eliminate structure VIII. The only difference between structures VII and VIII is that VII has the 3α while VIII has the 3β configuration. When the 3α and 3β isomers of 17α-methyl-Δ^4-androstenediol were chromatographed, they behaved identically. It is assumed that the 3β and 3α isomers of the anediol would also behave similarly. If this assumption holds, then the metabolite could not have structure VIII either.

The compounds represented by structures IX and X will be considered together. These structures also may be eliminated because when an Oppenauer oxidation is performed on compounds of this structure they are oxidized to a Δ^4-3-ketone. Phosphotungstic acid also reacts with these compounds to give a purplish red color. Since the metabolite did not react positively under the conditions necessary for the reactions to take place, it is evident that it does not have either structure IX or X.

From an examination of the evidence in this investigation, the metabolite is an alcohol because it will form an acetate. Due to the similarity in the movements of the acetates of the 17α-methyl-Δ^4-androstenediols and of the metabolite, it is very probable that the metabolite is also a diol.

The structures containing a ketone, and those containing a double bond at either carbon atoms 4 or 5 have been eliminated from the list of suggested metabolites. In addition, evidence excluding the androstane nucleus (structures VII and VIII) has been presented. Due to the process of elimination then, all of

the structures except V and VI have been discarded. At the present time there is no evidence against either of these structures. Therefore, the metabolite may be either the 3α or 3β isomer of 17α-methyletiocholane-3,17β-diol. Since a synthesis of these compounds has not been reported, no comparison of characteristics could be made.

II. Enzyme systems involved:

This work does not clearly indicate whether one or more enzymes are required for the reduction of the two groups in ring A. The lack of change in the chromatographic movement of the product produced by enzymatic action as more purified enzyme preparations were used, and the absence of a saturated ketone or a Δ^4 or Δ^5 unsaturated 3-ol, indicates that no separation of enzyme systems was achieved by any purification procedure used in this work. However, the radioactivity that was trapped in the DHT after an incubation of testosterone-4-C^{14} indicates that the major metabolite is probably formed by a two-stage reaction.

It was also noted that not only did the DHT trap some of the radioactivity but that the amount of unchanged radioactive testosterone remaining was larger when DHT was present. It appears, therefore, that DHT is competing with either testosterone or its intermediate for an enzymic site. If the competition were with the intermediate for sites on a second enzyme, an accumulation of the intermediate would be expected, as conversion of testosterone would only be reduced by product inhibition.

Since the completely reduced product appears to be an etiocholanediol, the two possible intermediates would be Δ^4-etiocholen-17β-ol-3-one or Δ^4-etiocholene-3,17-dione. There was no measurable radioactivity in the region where the latter compound would chromatograph, but accumulation of the former could not be ruled out since, without overrunning, it could not be distinguished from the final product. The other possibility would be that the DHT competed directly with testosterone for the original enzyme site, and that both reductions took place on the same enzyme. In this case, there would be no accumulation of intermediate.

When the specific activity of DHT is compared with that of the major metabolite formed from the incubation of radioactive testosterone, approximately 10 times as much is found in the metabolite. This situation could be explained by assuming that both reductions are usually carried out before the product is released for the first time from the enzyme surface after the attachment of the substrate. Since the DHT is present in excess amounts, it could displace some of the labeled intermediate from the enzyme thereby leaving some radioactivity in the medium. By this mechanism a small amount of radioactivity would be found in the DHT while the majority of the radioactive molecules would be converted to the completely reduced product before they were released from the enzyme. Because there is no competition for the second stage of reduction when DHT is absent, a nondetectable amount of

intermediate would be all that is released into the medium. The trapped radioactivity in the DHT as well as the lack of formation of a radioactive intermediate in the absence of DHT can be explained by the above assumption.

Another explanation for the greater activity in the ultimate metabolite is that DHT is not the major intermediate formed from testosterone. If the compound formed from testosterone has the 5β rather than the 5, configuration, then it is probable that the 5β compound would be much the better substrate for the second enzyme. If this explanation is the right one, the ratio of molecules reduced would favor the ones having the 5β configuration; thus a higher specific activity would be found in the major metabolite.

The evidence is more in favor of a two enzyme catalyzed reduction. However, there is no evidence that cannot also be explained so that it will apply to a one-enzyme catalyzed reduction. While there is no precedent established indicating that one enzyme is capable of catalyzing the reduction of a ketone group and a double bond, some enzyme preparations, thought to contain a single component, catalyze binary reactions. Talalay (101) has isolated a bacterial enzyme that will reduce the ketone groups on both C_3 and C_{17} of Δ^4-androstene-3,17-dione. Phosphoglyceric aldehyde dehydrogenase will oxidize the aldehyde group to the acid as well as form an ester with phosphate. Both the C-N and the C-O bond can be hydrolyzed by carboxypeptidase. The enzyme investigated here

may also catalyze a binary reduction or a technique capable of separating the enzyme system into more than one component may not have been used.

The occurrence of two temperature optima also points toward a two stage system. If, however, the product of one reaction were the obligatory substrate for the other reduction, an accumulation of this intermediate would have been expected at one or the other temperature optimum. Although the reduction of methyltestosterone at either optimum was 0.2 micromole or more per flask, no evidence of the presence of an unsaturated diol or of a saturated dione could be found; only the spot acting like a saturated diol was present. If the two temperature optima are associated directly with the two steps in reduction of the steroid, either reaction would appear to occur independently of the other, and the capacity of both systems must be adequate to carry out the second reduction of either intermediate.

Since, however, the enzyme systems used in the temperature studies were not pure, and the source of cofactor was boiled liver extract, it may be that one temperature optimum is that of an enzyme involved in the production of DPNH or TPNH, and that the concentration of cofactor became limiting at certain temperatures. In this case, one could assume that both reductions are taking place at the same enzyme site.

The enzyme systems studied here may be similar to those described by Tomkins (85) which catalyze a reduction of ring A of the corticosteroids. He claims to have separated his

system into two components. One initially reduces the double bond and the other reduces the 3-ketone group. He has been able to accomplish this separation by calcium phosphate gel adsorption. Contrary to his results, no indication of a separation of the enzyme system responsible for the saturation of ring A of methyltestosterone or testosterone was achieved in this work when calcium phosphate gel adsorption was employed. This discrepancy may be the result of differences between calcium phosphate gel preparations in mixing, aging, or other factors involved, since there is evidence that calcium phosphate gel is sometimes erratic.

If two enzymes were necessary for the production of a saturated ring A alcohol, then some enrichment of one of the enzymes over that of the other would have been expected during the purification procedure. No evidence of this was found. Some change in the ratio of the activities of the two enzymes could have been obtained and still been undetected, provided the second enzyme was more active than the first one. This situation would account for the failure to detect any intermediate as long as the second enzyme was present in sufficient amounts to catalyze almost completely the reduction of the intermediate formed. In this case there would always be an undetectable amount of intermediate present. The 3α-hydroxysteroid dehydrogenase described by Tomkins (86) was shown to be more active than the enzyme catalyzing the reduction of the double bond. If the enzymes under investigation exhibit the same characteristics, then an enrichment of one enzyme could

have taken place without detection.

The only cofactor shown to be necessary for the complete saturation of ring A is TPNH. If two enzymes are necessary for the reduction then both of them must be able to use TPNH. Tomkins (85) has found that TPNH is the only cofactor that will function in the reduction of the double bond but that either TPNH or DPNH will act with his 3α-hydroxysteroid dehydrogenase. It may be that the same arrangement applies to this system.

The evidence of this investigation does not decisively indicate whether the enzyme system studied in this work is similar to, the same as, or a different system from that described by Tomkins (85) although certain similarities of the two have been mentioned.

In view of the inhibition found when p-chloromercuribenzoate was present, and the destruction of enzymatic activity during incubation in an O_2-CO_2 atmosphere, it seems probable that one enzyme site has an essential -SH group. It is rather unusual, therefore, that no activation or protection of the enzyme was found in the presence of cysteine. However, some investigators (102) were only able to show a cysteine activation or protection of an enzyme which had been highly purified. It may well be that this enzyme has not been obtained in a state of purification high enough for this effect to be noticed. Otherwise, no reason has been found to explain the ineffectiveness of cysteine in this respect.

It is rather enticing to speculate on the structure of the

substrate required for attachment to the enzyme catalyzing the first step. The results from the study of various steroids indicates that a substituted 17 position increases the affinity of the enzyme for the substrate. However, the relatively high activity of the compound Δ^4-androstene-3-one having no substituted group on the 17 position, indicates that this point of attachment is not of great importance. Since the methyl groups at positions 10 and 13 both are sterically situated on one side of the steroid nucleus these groups may play a major role in the binding of the substrate to the enzyme. Whether any configuration of the steroid other than the Δ^4-3-ketone grouping of ring A is important for the enzymatic reactions can be only speculated on at the present time.

It appears that approximately one-fourth of the hydrogens transferred to the steroid molecule on reduction of the A ring are donated by the medium. Whether or not this is an obligatory condition cannot be elucidated from the information obtained in this investigation. Loewus et al. (103) have shown that the deuterium from the medium is transferred to the malate during the reduction of oxalacetate if the oxalacetate is in the enol form. In contrast to this, they concluded that all of the hydrogens transferred to the keto form were donated by DPNH. Since approximately one-fourth of the hydrogens acquired are donated by the medium to the steroid when testosterone is reduced in the system studied here, it is not unlikely in view of the results of Loewus et al. that the two hydrogens required

to reduce the ketone group and one of the hydrogens necessary to reduce the double bond are donated by TPNH. As a result, a strong nucleophilic center would exist around the other carbon atom. Hydrogen ions from the medium would then be attracted to this center and attached to the carbon. This mechanism would account for the results obtained.

Another explanation, however, may be offered for these results. Talalay et al. (104) found a transfer of deuterium from the medium of testosterone when Δ^4-androstene-3,17-dione was reduced by a bacterial enzyme requiring DPNH. This transfer was apparent only when a fairly crude enzyme preparation was used. When a more highly purified enzyme was employed all of the hydrogens transferred to the steroid were donated by the DPNH. An explanation for this finding was not offered. It may be, therefore, that the same mechanism may apply to this work and that a more purified enzyme would transfer the hydrogens solely from TPNH to the steroid.

IN SUMMARY, it may be stated that rat liver contains an enzyme system which reduces the Δ^4-3-ketone group in ring A of 17α-methyltestosterone and other C_{19} and C_{21} steroids forming a product that probably has the 5β-configuration. This reduction is probably carried out in two stages. The first stage appears to be a reduction of the double bond, since DHT acts as a competitive substrate for the second stage of the transformation. Since no intermediate products have been isolated during incubation with any enzyme fraction used,

it appears that the second stage is much more active than the first. Because TPNH is the only required cofactor that has been found, both enzymes, if two are required, must be able to use it. The enzyme catalyzing the first stage also appears to have a sulfhydryl grouping which is necessary for enzymatic activity. At the present stage of purification some of the hydrogens used to reduce the steroid A ring are donated by the medium. The probability remains that this system may be the same as that studied by Tomkins.

SUMMARY

Methyltestosterone has been incubated with rat liver preparations. The metabolite found in the largest concentration was more polar than 17α-methyltestosterone. It was radioactive when 17α-methyltestosterone-20-C^{14} was incubated. No double bond or ketone groups have been demonstrated on the molecule. The steroid was capable of forming acetates indicating it was an alcohol. Essentially no radioactivity was lost during incubation which implies that the methyl group at position 20 was not lost during metabolism. This metabolite moved different from 17α-methylandrostane-3α,17β-diol during chromatography. By the process of elimination of the possible reduced compounds, the metabolite was tentatively identified as either the 3α or the 3β isomer of 17α-methyletiocholanediol.

Other radioactive substances have been isolated when 17α-methyltestosterone-20-C^{14} was incubated. These substances have a polarity similar to that of the corticosteroids. Further identification has not been carried out on these metabolites.

The enzyme or enzymes responsible for the reduction of ring A have been found in the supernatant solution from 100,000 x g. centrifugation. The only cofactor which had been demonstrated to be required for complete saturation of ring A was TPNH.

Attempted purification of the enzymes has been tried by using ammonium sulfate fractionation, calcium phosphate gel adsorption, ethanol fractionation at reduced temperatures, and a second ammonium sulfate fractionation. Although an increased quantity of steroid reduced per mg. of nitrogen incubated was found during purification, no evidence of a separation of the enzyme systems was indicated. The major product isolated from all of the fractionation procedures by chromatography appeared to have the same characteristics. No evidence of an intermediate ketone or Δ^4 or Δ^5-3-ol was found.

The enzyme was found to be fairly labile to a change of time, temperature, and pH.

p-chloromercuribenzoate completely inhibited enzymatic activity. Approximately 50 per cent inhibition by KCN was found with the high concentrations used.

Although a sulfhydryl group seemed to be involved, no activation or protection of the enzyme was found in the presence of 0.005M cysteine.

A number of steroids having a Δ^4-3-ketone grouping in ring A and having different groups on position 17 were found to be substrates of the enzyme. Testosterone was shown to be the most active substrate of those that were used.

Some radioactivity was found in the dihydrotestosterone isolated when testosterone-4-C^{14} was incubated in the presence of dihydrotestosterone. However, its specific activity was about one-tenth of that of the major metabolite.

Kinetic measurements of the enzyme fraction obtained at 50 per cent of ammonium sulfate saturation but not at 40 per cent show two temperature optima and indicate two pH optima. The calculated K_m was 2.64×10^{-5} M.

By the use of tritiated water, it appears that approximately one-fourth of the hydrogen ions required for the reduction of the double bond and ketone group in ring A are donated by the medium at this stage of enzyme purification.

Whether or not one or more enzymes are required for the saturation of the double bond and the ketone group on ring A is discussed.

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